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CANCER RESEARCH

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CANCER RESEARCH

A MONTHLY JOURNAL OF ARTICLES AND ABSTRACTS REPORTING CANCER RESEARCH

VOLUME 7 OCTOBER, 1947 NUMBER 10

Degenerative Changes Induced in Tumor Cells by *Serratia marcescens* Polysaccharide*

Irene Corey Diller

(From the Lankenau Hospital Research Institute, and Institute for Cancer Research, Philadelphia 30, Pennsylvania)

(Received for publication May 12, 1947)

Preliminary reports of these investigations by Diller and Shear (9) and Diller (8) indicated in a general way the degenerative responses of tumor cells to a polysaccharide derived by Shear and his associates (18) from *Serratia marcescens* culture filtrate. The present paper comprises the detailed account of these cellular changes.

The use of bacterial toxins in tumor therapy was reviewed by Shear (15, 17) and more recently the application of bacterial agents in the form of Coley's "mixed toxin" to human neoplasms was surveyed by Nauts, Swift and Coley (13). However, the literature reveals no more than superficial notice of the effects of such agents on tumor cells. Apitz (4) in 1933 noted that there is an edematous appearance of tumor cells following toxin treatment, which may be separate from the anoxia caused by hemorrhage; and Andervont (3) in 1936 reported that treatment with meningococcus and *B. coli* filtrates causes tumor cells to swell prior to hemorrhage "indicating the bacterial products may have some direct effect on tumor cells."

This hypothesis is supported by a microscopic study, made over a 2 year period in our laboratory[1] of the effects produced by Shear's polysaccharide on the tumors of rats, hundreds of mice, and some hospital patients.

MATERIAL AND METHODS

Most of the studies were carried out on transplantable mouse sarcoma 37, supplemented by spontaneously occurring rat and mouse carcinomas and by human biopsy material from 16 sarcoma patients.

Small pieces of each tumor were routinely smeared for rapid examination in acetic-orcein, while the remainder was preserved in a modified Bouin's fixative (1.5 gm. of urea added to the standard formula) so that when supplementary data were needed, paraffin sections could be prepared for orientation studies and pathologic diagnosis. For demonstration of cytoplasmic components and archoplasmic structures, the coverslip smear technic described by Diller (7) was used.

The hosts for transplanted tumors were Carworth Farms albino mice (usually males) approximately 3 months of age. No difference could be noted between males and females with regard to polysaccharide response. Animals with well-established 6 to 10 day tumors which had not perforated the skin were chosen for treatment. Tumors open to the surface tend to be polysaccharide resistant, as noted by Shear and his associates (18), owing probably to the presence of infectious organisms that enhance immunity. Actively proliferating 7 day tumors arising from fragments implanted with a No. 12 trochar in a dorsal position are flattened ovals at least 7 to 10 mm. in their longest dimension, and tumors that had not attained approximately this size were discarded.

The standard mouse dose of polysaccharide employed for the experiments was 0.01 mgm. in a volume of 0.1 cc. of sterile saline, injected intraperitoneally. Other amounts employed are mentioned specifically. Intravenous injection in mice did not alter the course of cytologic response, and the simpler intraperitoneal method was therefore used. The material is highly toxic, and the amount stated usually caused death within 24 hours for 2 to 3 out of every 10 animals treated. Frequently death occurred at 4 to 5 hours, before the maximum cellular response could be realized. At higher dose levels, animals sometimes continued to suc-

*Reproduction of illustrations aided by a grant from funds contributed in memory of Paul Kessel and Paul Kessel, 3rd.

[1]Part of a joint study of tumor chemotherapy conducted by the National Cancer Institute and the Lankenau Hospital Research Institute.

cumb as late as 48 hours after administration of the polysaccharide, but when death followed a 0.01 mgm. injection, it occurred most frequently during the first night after treatment.

RESULTS

Effects on Tumors in Animals

Hemorrhage production in sarcoma 37 following polysaccharide administration has been described by Shear (17) and within the limits of individual variation, similar phenomena appeared during our experiments. Cytologically, individual tumors are not equally responsive, a fact which may be dependent upon the number of dividing cells present and the growth pattern of the tumor, as will be discussed subsequently. Microscopic observations indicate that cellular damage is produced in tumors showing little or no hemorrhage, as well as in those in which hemorrhage is extensive, but many tumors are only partially affected. However, no tumor thus far examined failed to respond to a greater or lesser degree by cellular degeneration, whether or not there was gross hemorrhage. In general, the areas of the tumor which were in closest proximity to the blood supply were first and most drastically affected.

For our cell studies, tumors were examined postinjection at hourly intervals, beginning with ½ hour after treatment. At 1½ to 2 hours the first cellular responses appeared in sarcoma 37, whether or not there was macroscopic hemorrhage. Prophase nuclei were primarily affected and reacted by forming surface blisters, or blebs, which were sometimes minute, at other times were more extensive, and in extreme cases there were multiple blebs. (Figs. 2, 3, and 27). In the last case, the entire nucleus appeared to have swollen, and at times pseudopodial processes (Fig. 3) were thrust out.

From 2 to 2½ hours, fewer nuclei with blistered membranes were encountered. Instead, many nuclei were shrunken, as though by withdrawal of fluid. As revealed in paraffin preparations, there was a corresponding shrinkage of the cytosome, which resulted in the disruption of the tumor tissue through separation of the cells into discrete entities. Resting nuclei were in most instances morphologically unchanged; occasionally, however, they were enlarged through apparent imbibition of fluid, but no blistering of their membranes was observed.

Correspondingly, those nuclei which were in metaphase at 2 hours after treatment showed considerable damage (Fig. 9). A series of changes appeared to have taken place between 2 and 3 hours which involved various aberrations in nuclear structures. Some were slight, *e.g.*, the displacement of individual chromosomes with respect to the metaphase plate; others showed drastic disorganization such as that in Fig. 10. Furthermore, there was a loss of stainability, which caused the center of the chromosome to appear hollow and vesiculate, although the outer rim was heavily stained. Sometimes these transparent bodies had become confluent and produced enlarged hyaline structures like those of Fig. 5. At other times, coagulation involved all the chromatin, as in Fig. 7.

Nuclei that arrived at the anaphase before this period were apparently successful in completing division, for there was no evidence of anaphase or telophase degeneration; therefore, polysaccharide did not appear to suppress division through any action on the spindle, as is the case with colchicine. At 2 to 3 hours after treatment, cell division was still uninhibited; though a considerable number of prophase nuclei showed surface modifications, and numerous metaphases were disrupted.

Preparations made between 3 and 3½ hours postinjection indicated a more advanced stage of degeneration involving pycnosis of large numbers of nuclei and considerable shrinkage of many others. These nuclei appeared more concentrated with decreasing size and assumed a bean or kidney

DESCRIPTION OF FIGURES 1 TO 14

Camera lucida drawings from acetic orcein smears (unless otherwise indicated) of sarcoma 37 treated with 0.01 mgm. of *S. marcescens* polysaccharide. Mag. × 2,500 (approx.).

Fig. 1.—Untreated prophase nucleus.

Fig. 2.—Prophase nucleus of teraploid cell (4 nucleoli) 2 hours after treatment. Note blistering of nuclear membrane.

Fig. 3.—Prophase nucleus, 2 hours after treatment; note shrinkage of nucleus and production of pseudopodial process.

Fig. 4.—Prophase nucleus, 3 hours after treatment.

Figs. 5, 6, and 7.—Metaphase nuclei, 2 hours after treatment.

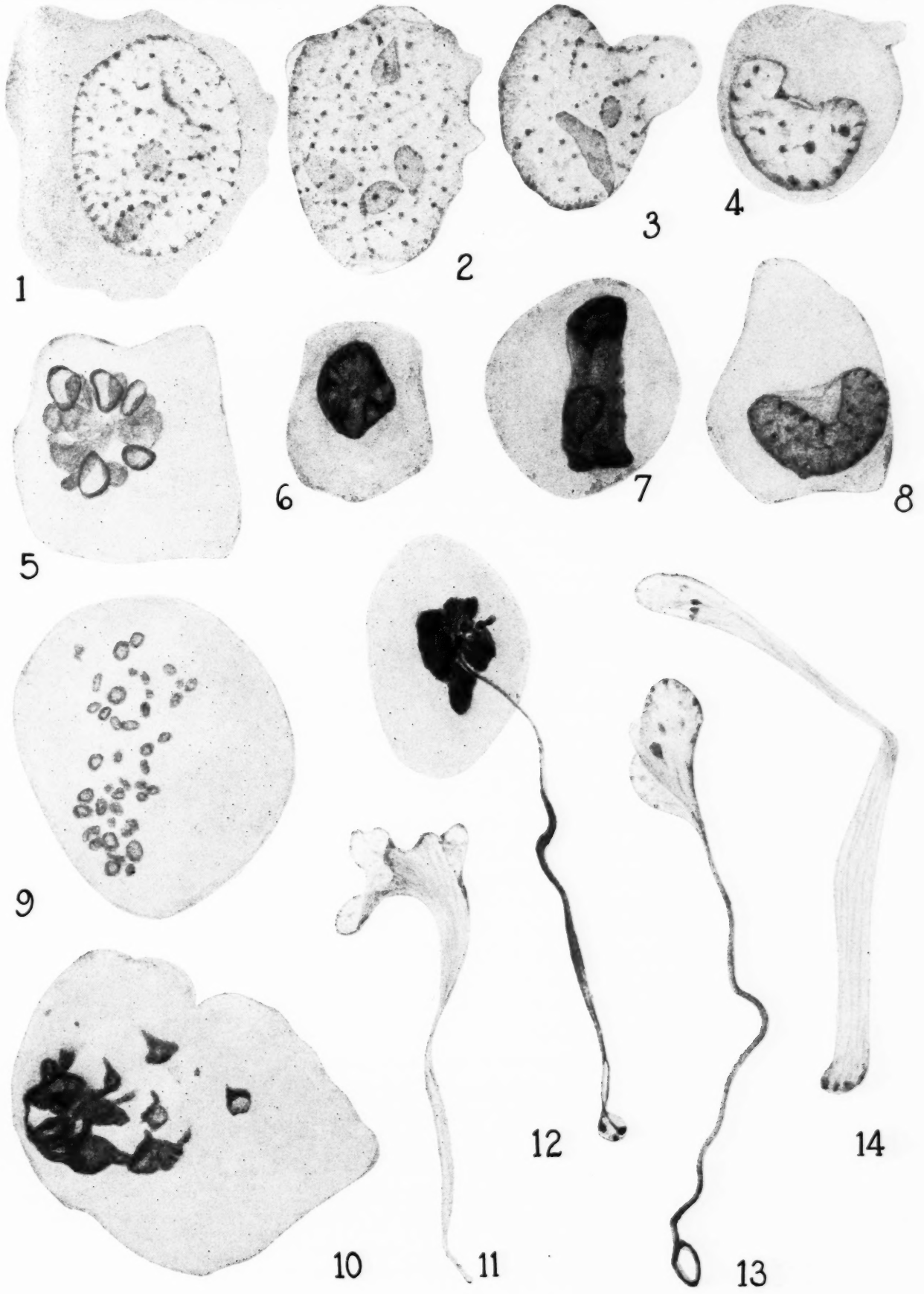
Fig. 8.—Shrunken nucleus, 3 to 4 hours after treatment.

Figs. 9 and 10.—Metaphases, 3 to 4 hours after treatment.

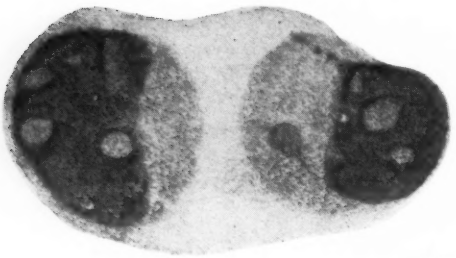
Fig. 11.—Extrusion of nuclear filaments, 4 hours after treatment.

Fig. 12.—Extrusion of a single longitudinally divided chromosome from metaphase clump 4 hours after treatment (paraffin preparation, Feulgen stain).

Figs. 13 and 14.—Distorted nuclei with extruded filaments, 4 to 5 hours after treatment (paraffin preparation, hemalum stain).



FIGS. 1-14



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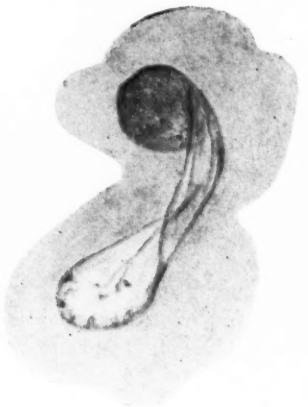

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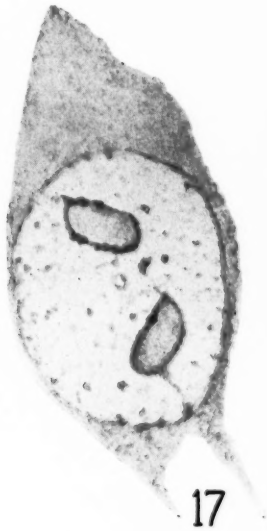

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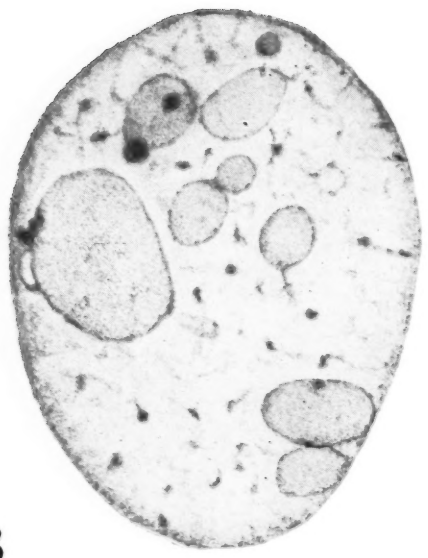

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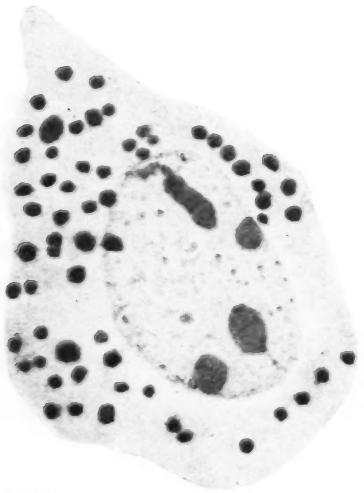

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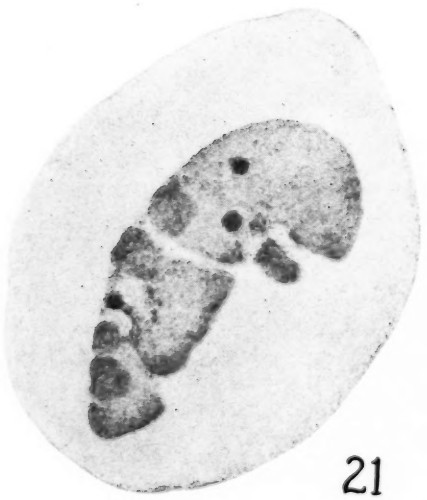

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FIGS. 15-22

shape (Figs. 4 and 8). Whether these always arose through collapse of previously swollen nuclei, it was not possible to judge. Stages indicating progressive shrinkage of nucleoli were likewise demonstrable, and these bodies ultimately came to be represented only by dark granules that took orcein stain, but no fast green. The cell body also decreased in size and was more heavily stained. Blebbed nuclei could also be found. Metaphases were practically all degenerative, *e.g.*, Fig. 6 (see also Figs. 30 and 31), and there were no anaphase or telophase figures. Sister cells which were in the process of separation at 1½ hours apparently succeeded in completing division, but thereafter the division process usually did not go beyond the metaphase. In many tumors studied the only normal cells visible at 3 hours post treatment were resting ones. Some of the tissues showed large areas in which the orcein, staining the nucleoproteins, was dispersed throughout the cell instead of being limited to chromatic structures. Furthermore, there appeared at about this time nuclear disruptions so drastic that they must certainly be irreversible. They were the most characteristic feature of polysaccharide response.

The essential feature of this change was the extrusion from the nucleus of filamentous orcein-positive (chromatin) bodies, sometimes involving many threads and sometimes consisting of a single chromosome pair (Fig. 12). Here the chromatin material of what is apparently a degenerating metaphase figure is clumped into a formless mass for which the longitudinally double chromonemata of a single pair of chromosomes have been extruded far beyond the confines of the nucleus, and even of the cell body, to attain an extended dimension approximately 4 times that of the clumped nucleus. This figure (shown also in Fig. 32) is from a paraffin preparation subjected to the Feulgen reaction. The greatly extended thread is Feulgen-positive and still exhibits some faint traces of residual coiling. A dimly discernible body attached to the pycnotic distal granules appears to be the remnant of the nucleolus.

A similar attenuation involving remnants of prophase nuclei appears in Figs. 11 and 13. Even more distorted nuclei filled with fairly individualized chromatin threads drawn out to a considerable length are also present (Fig. 14). These figures are better demonstrated in smears than in paraffin sections, where they are so long that they can seldom be found in one section but appear in cross section as chopped-up fragments. It is barely possible that the pressure exerted in making the smear preparation may tend to exaggerate the effect; but the same phenomenon is apparent to a lesser degree in paraffin preparations, where the tendency is in the other direction, toward shrinkage and a consequent masking of the early effects of the polysaccharide.

At 4 hours practically all metaphase nuclei were clumped (Fig. 8) whether or not there were chromatin extrusions from the central mass. By this time some of the metaphases were nothing but pycnotic spheres, sometimes perforated by vacuoles (Fig. 6). No normal metaphases, and no telophases at all, persisted. Such intact cells as survived were in resting stage, but they often appeared greatly swollen and contained huge, correspondingly swollen nucleoli. Blebbed and reniform nuclei were also present.

A different kind of degeneration of the tumor sometimes occurred about 4 hours postinjection. This involved a complete coagulation of both nucleus and cytosome (Fig. 24).

Between 4 and 5 hours when tumors grossly exhibited considerable hemorrhage, degeneration of initially affected cells neared its peak. Swollen resting nuclei, blebbed prophase nuclei, bean-shaped, shrunken nuclei, clumped metaphases and extruded filaments were all present. The shrunken prophases without extruded filaments had no semblance of ordered form and became twisted "ghosts" from which cell boundaries were lost, while the pale, muddily stained nuclear remnants lay upon an amorphous mass of degenerating cytoplasm. Individually these bodies rounded into spheres of descending sizes or faintly stained bodies of irregular contour, from which all trace of structure had disappeared. The spherical bodies apparently arose from metaphase degenerates, while twisted, folded, and flattened figures were

DESCRIPTION OF FIGURES 15 TO 22

Camera lucida drawings; smear preparations of sarcoma 37, treated with 0.01 mgm. of *S. marcescens* polysaccharide. Mag. × 2,500 (approx.).

FIGS. 15 and 16.—Degenerating telophases, newly dividing cells, 3 days after polysaccharide treatment (acetic orcein stain).

FIG. 17.—Undamaged resting nucleus, surviving polysaccharide treatment (acetic orcein).

FIGS. 18 and 19.—Nuclei, enormously enlarged in resting cells, during 3 day period following polysaccharide treatment (acetic orcein).

FIG. 20.—Resting nucleus during 3 day period of mitotic inhibition following polysaccharide administration (smear, Flemming fixation, Flemming tricolor). Note "fatty degeneration" of cytoplasmic substance.

FIGS. 21 and 22.—Degenerating nuclei from cells treated with polysaccharide and x-ray, 24 hours after irradiation.

remnants of nuclei in pre-metaphase stages. Degenerating blood cells were also present (Fig. 36).

The amount of destruction varied considerably, but in some tumors deterioration was so extensive that by 6 hours there was practically complete necrosis; and nothing remained but amorphous cytoplasmic debris with some nuclear fragments (Fig. 26). Recognizable cells were in an advanced stage of degeneration and seemed for the most part to be little more than distorted naked nuclei (Fig. 33).

Beyond this time (6 hours) the stages undergone by as yet undamaged cells were identical with those just described, and the result was merely an extension of the response to include greater areas of the tumor. The length of time during which degeneration can continue, and before any resistant cells are again able to resume division, is discussed in the following section.

Duration of effect.—As mentioned previously, the maximum hemorrhagic effect was reached at approximately 6 hours after injection of the polysaccharide. The tumors were usually encapsulated; and when extreme hemorrhage was induced, the neoplasm appeared on gross examination as a soft, bloody sac. It is not uncommon at this period to find such marked deterioration that at times no solid tissue is available, and little more than a bloody smear can be secured for microscopic examination. During this period the mice show lowered temperatures, accept no food or water, and remain huddled and immobile in their cages. Some casualties may occur from 4 hours onward, but survivors of the 0.01 mgm. dose usually completely recover within 24 hours. They were eager for food and could run and swing on their cages in a normal way, indicating that recovery from the toxic effects was quickly accomplished. In order to determine whether the cellular response of the tumors is correspondingly brief, any persisting residue of undestroyed tissue was studied at 24 hour intervals, up to 5 days postinjection.

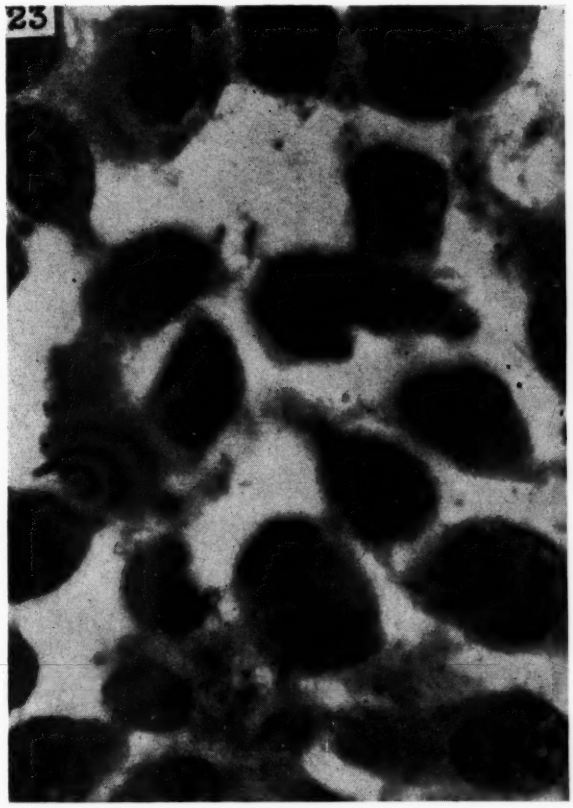
For this series of studies tumors were chosen, which, because of extensive hemorrhage, showed that they had reacted. As Andervont (3) demonstrated in connection with tumor response to meningococcus and *B. coli* filtrates, tumors which are totally hemorrhagic usually form a hard mass of dried blood beneath the skin within 24 hours. In our 24 hour material, the entire tissue was sometimes involved in the hardened mass, while at other times a certain amount of translucent tissue could be secured for examination. Invariably, in such material, the only seemingly normal cells were "resting" ones (Fig. 35). In the transition between interkinesis and the stage of active division the extreme diffusion of the chromatin substance made the distinction between "resting" and dividing nucleus difficult, if not impossible; but nuclei were arbitrarily judged to be in prophase when definite polarization of the heterochromatic proximal ends of the chromatin threads with respect to the nuclear membrane, together with resolution of definitive chromosomes, first became visible.

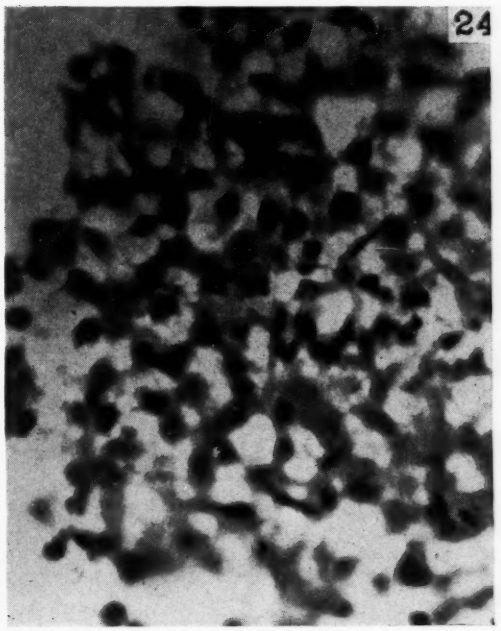
After 24 hours the only morphologically intact cells were those not visibly preparing for mitosis. There is some evidence that these cells, too, may respond in some way, through changes in the cytoplasmic constituents. An instance of this appears in Fig. 38. This photograph shows a single resting cell remaining undistorted amidst the debris of degenerating cells. The nuclear structures and cell membrane are intact, but the osmiophilic substance has undergone "fatty degeneration" (see also Fig. 20) and is massed in the cytoplasm as blackened spheres. Disrupted cells have extruded osmiophilic substance in the form of spherical masses, and these clumps, large and small, are scattered through the tumor 24 hours after polysaccharide administration.

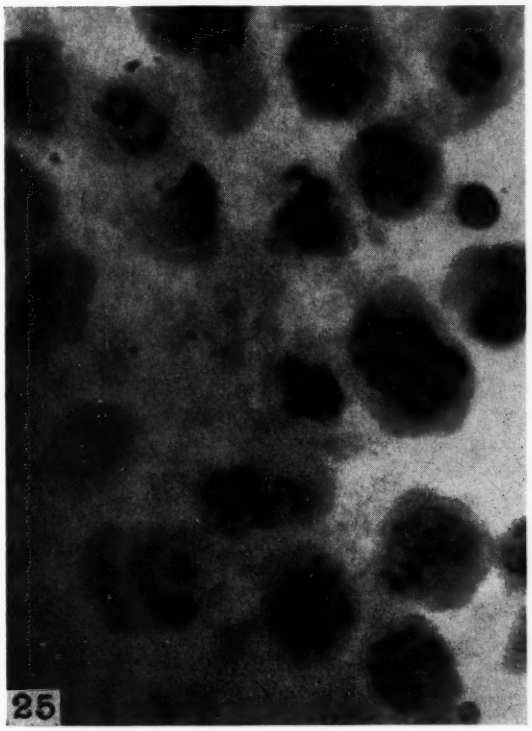
Degeneration of all types described in the previous section could be discovered at 24 hours, except in cases where all the material had reached the highly attenuated condition. Blood cells were present in large quantities. Erythrocytes by this time began to disintegrate and to confuse further the already chaotic picture by disrupting into amorphous masses and fragments (Fig. 37). Alternately, their substance, which is enucleate, was extruded in filamentous conglomerates resembling in the extremity of their attenuation the drastically elongated chromatic threads already described.

At 48 hours there still were no dividing cells. Resting nuclei appeared greatly enlarged, and judging by the number of nucleoli, which were tremendously swollen and frequently coalesced (Fig. 19), many of these were heteroploid. The necrotic areas now presented an appearance of complete chaos, in which all semblance of nuclear contour had disappeared, and only pycnotic bodies and fragments remained.

The first dividing cells were noted 72 hours after injection. Some of the divisions apparently succeeded, but many were abortive. Frequently, division was halted in metaphase with resultant pycnotic malformations and condensations, but the bulk of the cells degenerated at telophase. This did not involve spindle suppression or destruction but rather the failure of sister nuclei to





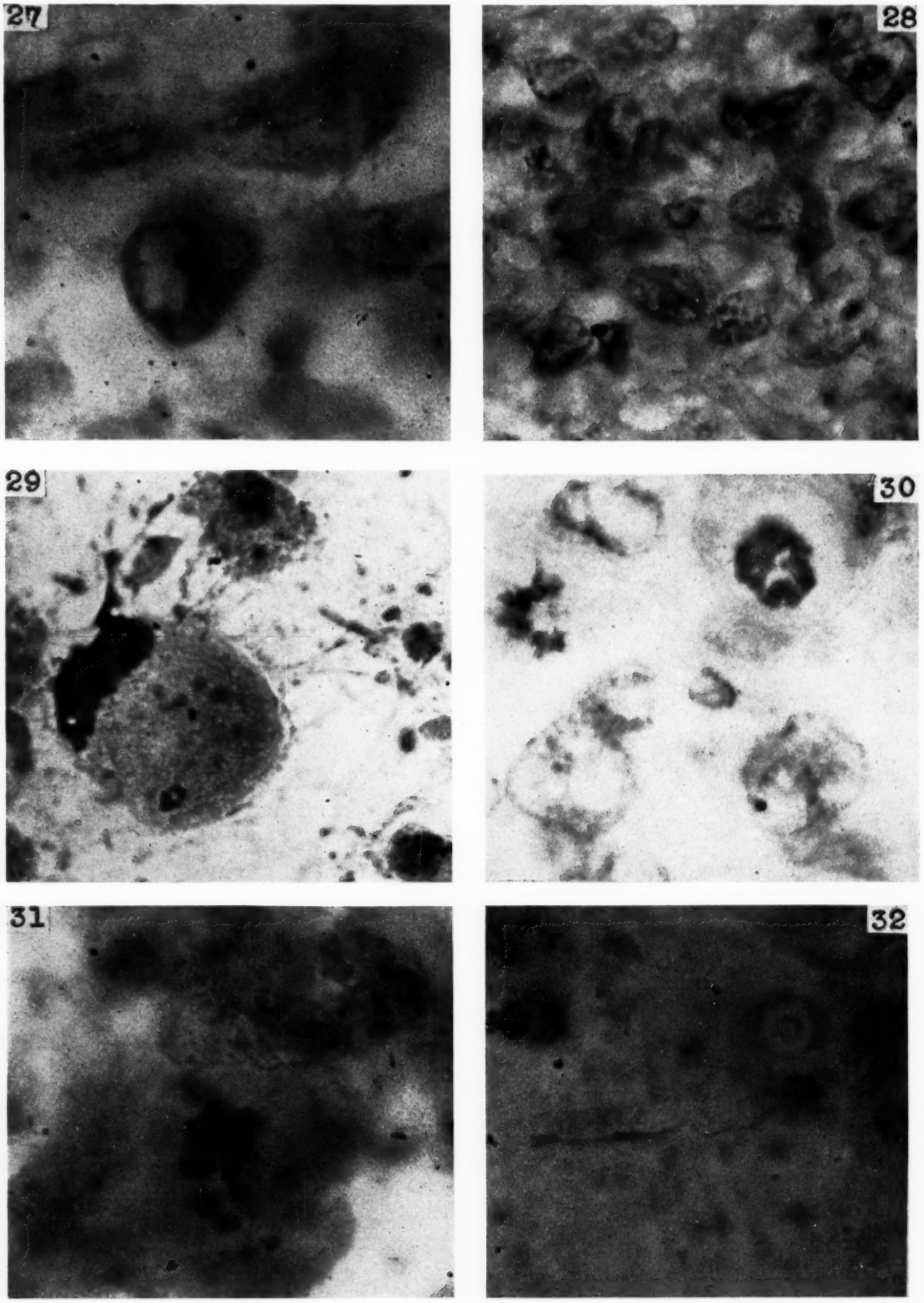


Photomicrographs from acetic orcein preparations of sarcoma 37, treated with 0.01 mgm. of polysaccharide.

FIG. 23.—Untreated control tumor. Mag. × 1,000.

FIGS. 24 and 25.—Tumor 4 to 5 hours after treatment. (Fig. 4, mag. × 720; Fig. 5, mag. × 1,000).

FIG. 26.—Same, 6 hours after treatment. Mag. × 1,000.



Photomicrographs from acetic orcein preparations of sarcoma 37, treated with 0.01 mgm. of polysaccharide. Mag. × 1,000.

FIG. 27.—Blebbing of nuclear membrane, 1½ hours after treatment.

FIG. 28.—Vacuolization of individual nuclei, 2 hours.

FIG. 29.—Metaphase degeneration in giant cell, 2 hours after polysaccharide.

FIG. 30.—One degenerating, and 1 normal metaphase, 2 hours after treatment.

FIG. 31.—Degenerating metaphase, 2 hours after treatment.

FIG. 32.—Same, 4 hours after treatment, showing extrusion of individual chromonemes. See also Fig. 12.

reconstitute resting cells (Fig. 16). The degenerates were frequently dumbbell-shaped figures comprised of persisting interzonal fibers or a connecting strand of nucleoplasm separating two pycnotic sister nuclei that tended to degenerate asynchronously.

If no resting cells persisted over the 3-day period the tumor was no longer viable, so far as was detected microscopically. No mitotic figures known to arise from the enormously swollen, obviously hyperploid nuclei were positively identified, but polyploid cells in every stage of division were numerous, as were also aberrant metaphases which were attempting regulation, *e.g.*, by throwing out supernumerary chromosomes or by formation of multipolar spindles. Quite possibly these are the products of the giant nuclei. Moreover, at this time it was not uncommon to find "nests" of dividing figures which might involve 6 or 8 metaphases superimposed upon one another in a single microscopic field, in a juxtaposition suggesting that they might be the products of a single giant cell.

By 5 or 6 days after treatment, tumors were again growing actively next to the body wall to an extent discernible even to the naked eye. Alternately, they were being sloughed off at the surface or shrinking down by resorption. The inhibitory effect of the bacterial substance on division processes in sarcoma 37 was, therefore, of no more than 3 days' duration. The first attempts at division were usually unsuccessful; but between 72 and 96 hours, morphologically unaltered resting nuclei were able to resume division and rapid proliferation of the tumor began.

The experiments thus far described were made on 7 to 10 day old tumors. Andervont (3) in discussing reactivity of mouse tumors to bacterial products states that the introduction of such substances "does not produce hemorrhage within skin tumors until the fourth or fifth day after inoculation, although growing tumor tissue is present as early as the third day." In order to discover whether there would be an accompanying failure of nuclear breakdown before the sixth day postimplantation, we studied a series of tumor implants treated with the usual amount of polysaccharide, at daily intervals, *i.e.*, 24, 48, 72, and 96 hours, and at 5 days after implantation. As we shall discuss later, onset of cell division in the tumor implant is probably correlated with availability of blood supply. It is evident upon gross examination that chance decides whether the implanted fragment comes to rest in close proximity to a vascular branch, or whether it must remain quiescent until capillary branching can be elicited, a 3 day process according to Algire's studies (2). This agrees with our own cruder observations and with Andervont's statement (3) that dividing tissue is present as early as 3 days after implantation.

In our own experience, proliferation of sarcoma 37 implants is correlated with establishment of contact with the blood supply. Tumor fragments fortunately placed in juxtaposition to capillaries begin to divide at the outer edges of the implant as early as 28 hours after deposition. On the other hand, fragments not in contact with existing blood capillaries may remain as long as 3 days without blood supply until capillary branches are established. Beyond this period they are apparently incapable of survival. In almost every case, only the part of the implant directly in contact with vascular branches enters into division; the residue degenerates.

Careful examination of many tumors reveals that when cells are dividing they will react typically to polysaccharide, regardless of the age of the implant. The same types of degeneration hitherto described (disrupted metaphases, shrunken crescents and filamentous extrusions) together with structurally normal resting nuclei, are found. Since nearly all the living tissue surrounding the young implant is in rapid division, it is not uncommon to find that 6 hours after administration of the agent to an animal bearing a 2 to 3 day implant, nothing remains except the opaque necrotic mass of implanted tissue which has not yet been absorbed, and a faint trail of blood where the capillary connections have been severed. In a few cases where the total implant had presumably become involved in division within 3 days (as indicated by the absence of any central necrotic plaque of tissue) no tumor tissue remained at the implantation site 6 hours after injection of 0.01 mgm. of polysaccharide.

Older tumors also reacted with the same type of cytologic change. Tumors were studied up to 16 days postimplantation, and although the conditions are more difficult to interpret because of the presence of large amounts of spontaneous necrosis, the expected types of damage were also encountered. Apparently a good many resting cells were dormant in these older growths, since they persisted in large numbers following polysaccharide treatment, and we could never succeed in breaking down a whole tumor but caused only a portion of it to react, as indicated by our microscopic findings and by partial sloughing macroscopically observable.

Effect of multiple or continuous treatments.— In about a fourth of the tumors examined, there was no resumption of division on the third day after

polysaccharide treatment; in the remainder, mitotic figures were plentiful. The obvious practical procedure would appear to be to reinject the host at 3 day intervals in the hope of suppressing each new mitotic wave. This experiment was tried, using the same amount and concentration of polysaccharide in each successive treatment until sloughing was induced or the animal succumbed to the tumor. Much to our disappointment, this induced no appreciable additional cell destruction; and growth, once re-established, was not noticeably impeded. Tumors treated over long periods (about 20 days) tended to become heavily encapsulated, and in only a few cases did we detect metastases. The failure of repeated doses of polysaccharide to suppress growth indicates that either the tumor or the host becomes resistant to the bacterial agent.

Since the amount of the original single injection could not be increased without causing the death of a still greater number of mice, we attempted to use polysaccharide of lower concentration in multiple doses of increasing magnitude. An initial injection of 0.001 mgm. in 0.1 cc. of fluid was administered, and thereafter the injections were increased by 0.001 mgm. daily until a total of 0.02 mgm. had been injected over a period of a week. Two series of tumors were treated simultaneously. One of these was examined microscopically on the seventh day after initial treatment, and the other series was allowed to run until the hosts either succumbed or were able to slough off or resorb the tumor. In the latter series, only 1 animal in 10 was able to rid itself of the growth. The others died within 20 to 24 days.

The tumors examined on the seventh day frequently were scabbed next to the skin and microscopically showed areas of degeneration in which no dividing tissue was present and which did not appear to arise from spontaneous necrosis. In these areas, the cytoplasm tended to be extremely viscous and coagulated. Even tumors exhibiting no gross hemorrhage but filled with clear, translucent tissue, had degenerative areas. In acetic orcein smears the affected portions were stained aberrantly, and the cytoplasmic substance, which should have been stained with fast green, was pinkish as though by diffusion of the chromatic substance from the nucleus. Where areas of dividing tissue impinged on degenerating ones, the dividing cells showed a high proportion of polyploid nuclei.

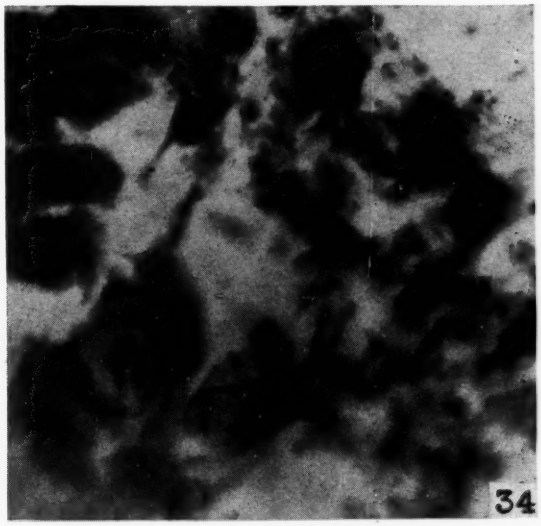
Also, we injected mice with single large doses (0.005 mgm. in 0.5 cc. of fluid) of the low concentration. At 5 to 6 hours there followed marked degeneration of all but resting nuclei, and there was total coagulation in the hemorrhagic areas. In the microscopic preparations, the tumor tissue showed clotting at the center, pycnotic rounded fragments, and heavily stained resting nuclei. At 24 hours, cell division had not been resumed in the affected areas. Nevertheless, tumors similarly treated and not removed for microscopic examination continued to grow and eventually caused the death of the host.

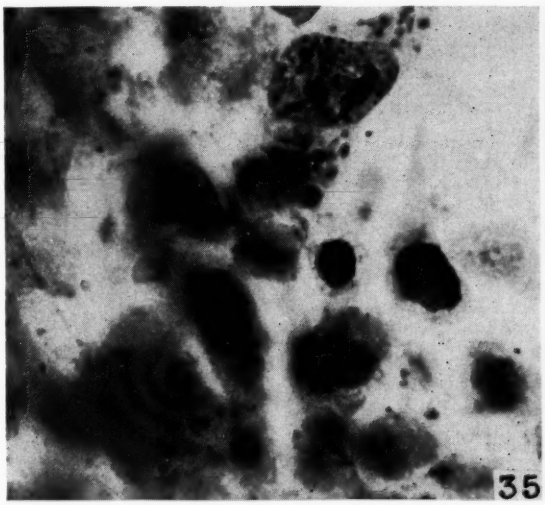
These experiments indicated that multiple doses of low concentration were not as effective in tumor-cell destruction as a single dose of a magnitude approximately ¼ lethal to the mouse hosts. Furthermore, multiple injections of larger magnitude did not offer much improvement over a single application in breaking down or inhibiting growth in tumor tissue.

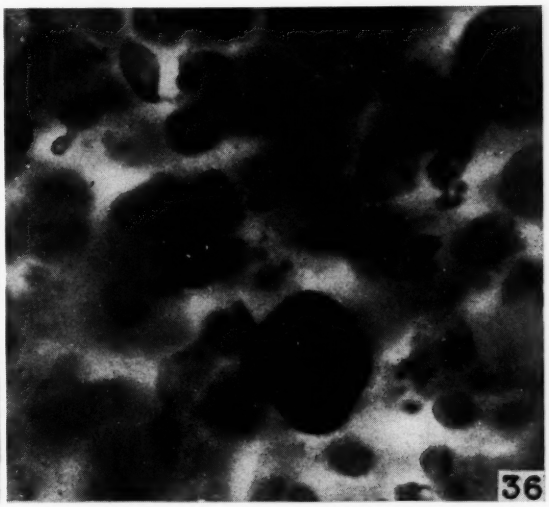
Injection directly into the tumor.—Since our studies showed beyond much doubt that polysaccharide has a direct effect on cells apart from that following hemorrhage, it occurred to us to try to obtain cell breakdown by injecting directly into the tumor with the hope that the toxic systemic effects produced in the host could thus be obviated. Injection of 0.005 mgm. of polysaccharide into a 7 day mouse tumor resulted in the disruption and bursting of some of the cells and, in one case, in a severe coagulation of the cytoplasm. Except for this instance there was not much difference in the appearance of cells from tumors so treated and control tumors injected with the same amount of salt solution or distilled water. In no case was division suppressed for more than a few hours and growth was at best only temporarily inhibited. This agrees with observations made in another department of this Institute on tissue cultures treated directly with polysaccharide (Royle, unpublished data), wherein the response was negative.

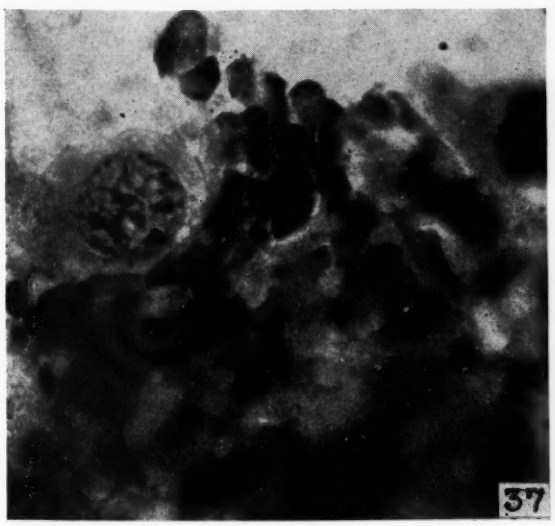
Effect of polysaccharide on normal body tissues.—Since the hemorrhage-producing effect of *S. marcescens* polysaccharide appeared to be confined to the region of the tumor, a study was made of normal tissues of treated mice to determine whether the tissue-destructive action of the bacterial product was likewise limited to tumor cells. So far as we were able to determine, this is not the case, although tumor cells did appear to be much more sensitive than nontumor cells, and response was elicited only in those tissues undergoing division or rapid nucleoprotein synthesis. Intestinal epithelium, which appeared to be the most actively dividing tissue in the adult mouse, was also the most reactive to polysaccharide.

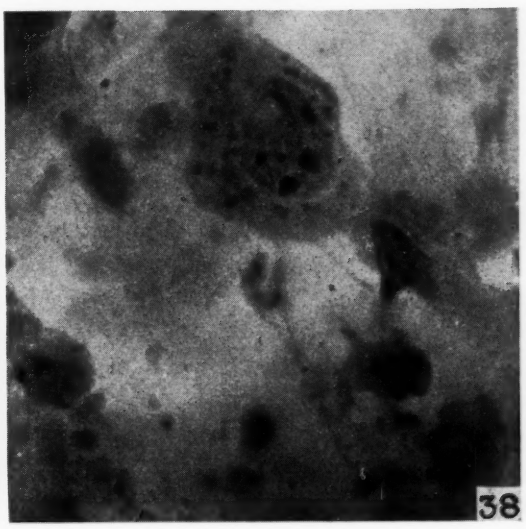
Following administration of polysaccharide, extensive filamentous degeneration was produced in the villi of the mouse intestine, which normally











Photomicrographs from smear preparations of sarcoma 37 treated with 0.01 mgm. of polysaccharide. Mag. × 1,000.

FIG. 33.—Tumor, 6 hours after treatment (hemalum-eosin stain).

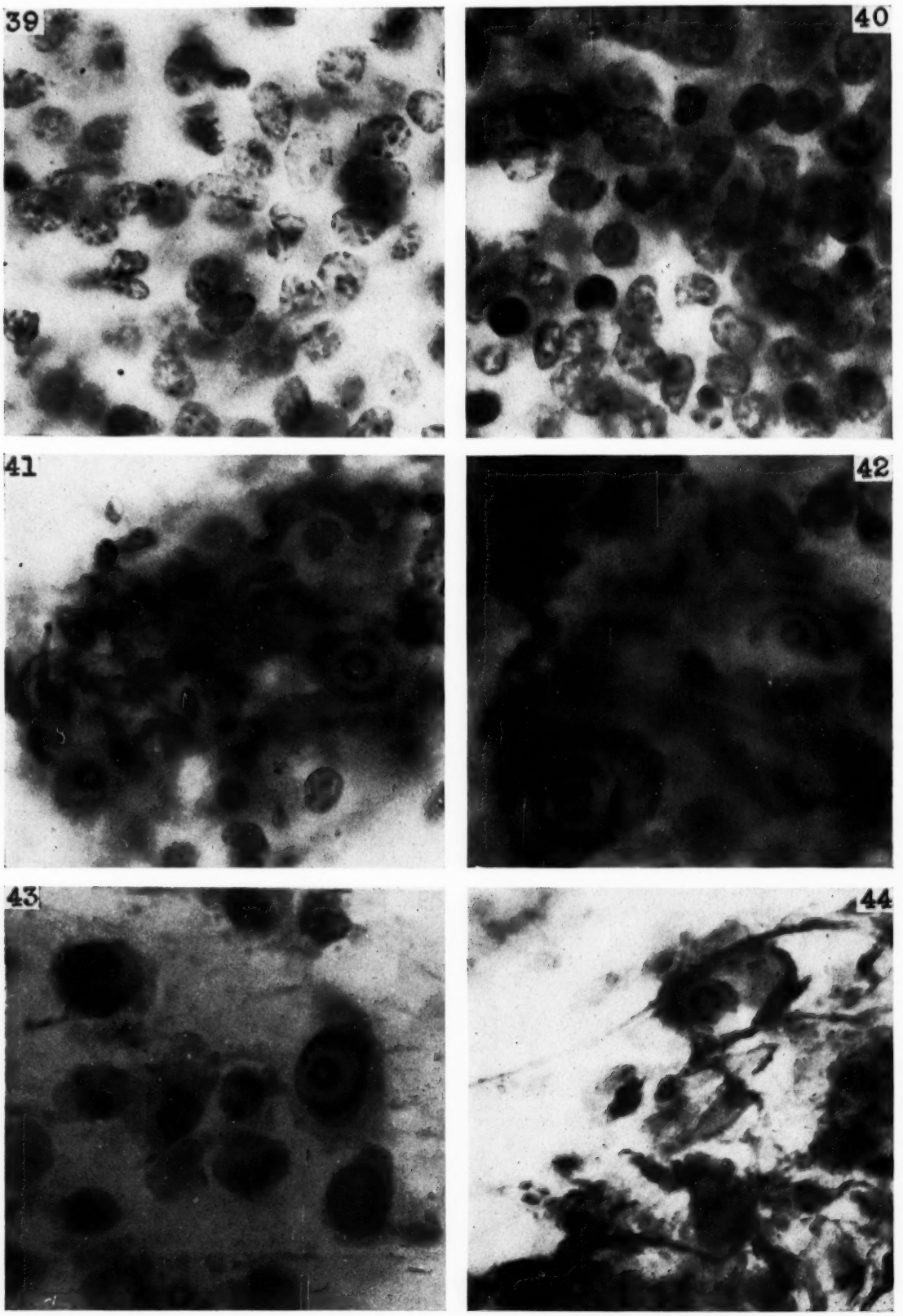
FIG. 34.—Same (acetic orcein stain).

FIG. 35.—Resting nucleus, morphologically unchanged, 24 hours after treatment. (Flemming tricolor).

FIG. 36.—Tumor 24 hours after polysaccharide treatment, showing degenerating blood cells (paraffin preparation).

FIG. 37.—Tumor 48 hours after treatment; resting nucleus and degenerating blood cells.

FIG. 38.—Three days after treatment; cytosome of resting nucleus filled with osmiophilic bodies which appear degenerative. See also Fig. 20.



FIGS. 39 to 44 Mag. × 1,000.

FIG. 39.—Spontaneous breast carcinoma of mouse, untreated.

FIG. 40.—Same, 6 hours after polysaccharide treatment.

FIG. 41.—Same, 24 hours after treatment.

FIG. 42.—Mammary carcinoma of rat, 48 hours after treatment.

FIG. 43.—Mouse sarcoma 37, 3 days after polysaccharide treatment (just before resumption of division).

FIG. 44.—Same, specimen from a totally nonviable tumor.

contained large numbers of cells in mitosis at all times. No difference was detected in the response of intestinal epithelium of tumor-bearing mice and those having no tumors. Fortunately, the amount of polysaccharide required to produce destruction of nontumorous tissue was greater than that required for degeneration for tumor cells. For instance, the usual dose of 0.01 mgm. produced only slight breakdown in intestinal epithelial cells, and in order to secure an effect comparable to that which would occur in a tumor following administration of the same amount of polysaccharide, a concentration 10 times as great (0.1 mgm.) was required. The reaction of intestinal epithelium to this amount of polysaccharide, 6 hours after intraperitoneal administration, appears in Figs. 46 and 48 (*see* Figs. 45 and 47).

Other tissues studied were spleen, bone marrow, kidney, liver, endocrine glands, and gonadal tissue. Of these, the damage was greatest in developing blood cells of the bone marrow and in the liver. The amount required to produce extensive damage was also 10 times that used for the destruction of tumor tissue. Kidney, which has few dividing cells, was almost completely resistant. A more puzzling phenomenon was the failure of response of gonadal cells, either spermatogonia or oogonia, to polysaccharide, even when an amount fatal to half the mice was employed. (Fig. 49). When tumor-bearing males and females, treated with polysaccharide in an amount sufficient to cause the sloughing of the entire tumor, were maintained for 2 or 3 months thereafter and bred *inter se*, normal litters of offspring were produced.

The effect of polysaccharide on the adrenal and other glands will be discussed in a later paper. A decided shrinkage of cells and nuclei of the adrenal, particularly in the medullary region, was produced within 6 hours after injection of 0.01 mgm., but the effect was usually reversible at this dose level within 24 hours.

Treatment of spontaneous neoplasms.—A series of studies of the reactions of mice with primary subcutaneous tumors to hemorrhage-producing polysaccharide was made by Shear (16). From his paper the following is quoted: "Sufficient evidence . . . has been accumulated to show that the production of hemorrhage and necrosis by such agents is by no means restricted to transplanted sarcomas. While it has been found by most workers that, in general, carcinomas are more refractory than sarcomas, nevertheless, several strains of transplantable carcinomas have been reported to be responsive to the action of these bacterial products. . . . Furthermore, this hemorrhagic and necrotic effect is not confined to transplanted tumors." The results of Shear's studies of 750 mice bearing chemically induced tumors show that, while administration of *S. marcescens* polysaccharide regularly produced hemorrhage and necrosis, some portion of the neoplasm usually escaped destruction and continued to grow progressively until the death of the host.

Spontaneous growths in rats and mice.—Cytological studies were made by us of the effect of polysaccharide on spontaneously occurring tumors that appeared in our stock colony of rats and mice, and also of a controlled series of mammary adenocarcinomas in C3H and dba mice. C3H mice with large mammary carcinomas, some of which were multiple, were treated in the same way as mice with transplanted tumors, *i.e.*, 0.01 mgm. of polysaccharide was injected intraperitoneally and the animals were killed at 6 hours. These mammary tumors were highly vascularized; and although treated tumors were scarcely more than sacs of blood at 6 hours posttreatment, we found the control tumors also to be highly hemorrhagic. Therefore the presence of a great deal of blood in the treated tumors could not in this case be taken as a criterion of polysaccharide reaction. However, microscopic comparison of control and treated materials revealed a definite cellular response which, though by no means as pronounced at 6 hours as in sarcomatous growths, was nevertheless striking (*see* Figs. 39 and 40). The extreme filamentous degeneration in sarcomas at 6 hours was lacking, but there was a rounding and pycnosis of nuclei.

A second series of mammary carcinomas in dba stock was treated with a larger amount of polysaccharide (0.02 mgm.) and the survivors were killed 24 hours after treatment. Considerable areas of the tumors were then found to be responding as in sarcoma 37, by extrusion of nuclear components (Fig. 41).

Spontaneous mammary carcinomas arising in our colony stocks of heterozygous white mice were also studied with similar results. Mammary carcinomas arising in aged albino rats maintained on butter yellow diets were also studied. Pretreatment biopsies were taken and injections of 1 cc. each of polysaccharide of low concentration (*i.e.* 0.01 mgm. of polysaccharide) were made into the rats. The animals were killed the following day and the tumors prepared by the paraffin method. Definite and typical, though not extensive, nuclear response was detectable, but the most interesting finding was that, although the capillaries penetrating into the neoplasm were still intact, the tissue immediately adjacent was highly degenerative.

Effects on Tumors in Human Beings

The human material available for study was obtained from biopsies taken of 16 sarcoma patients before and after treatment at the Lankenau Hospital during the winter of 1944-45. The clinical responses of these patients and the pathological findings were reported by Holloman (10) and by Oakey (14). Four patients had previously been treated with *S. marcescens* polysaccharide at another hospital, as described by Brues and Shear (6). The biopsy material available for our studies included specimens of chondrosarcoma, fibrosarcoma, lymphosarcoma, recurrent melanoma, chronic granuloma, secondary spindle-cell sarcoma, and nodes from the neck region of a patient suffering from Hodgkin's disease. The previous finding that carcinomas do not respond as readily as do sarcomas restricted the choice of cases for study to those with sarcomatous growths.

The course of cytological response followed the pattern already exposed by our studies of animal tumors. It differed only in the relatively small amounts of tissue affected. This was to be expected because the amounts of polysaccharide administered to human beings were relatively small in proportion to body weight compared with the doses employed in treatment of mice. As the previous clinical reports describe (10,14) human beings responded with much greater intensity than did mice, as far as toxic effects were concerned, and for this reason in the preliminary trials the amounts used were minute, *i.e.*, 0.01 mgm. in a volume of 1 cc. administered intravenously. Our studies with mice had shown that in sarcoma 37 the ultimate cytological response was the same regardless of the route of injection. The cellular conditions were so similar in all the sarcomas studied that it is not necessary to present examples of every case, but the findings on 4 different types of human neoplasm are presented. Figures 51 to 54 are photographs from paraffin preparations used in pathological diagnosis and are typical of the general histological picture following polysaccharide treatment. The remainder of the figures are from acetic orcein smears prepared especially for nuclear study.

Fibrosarcoma.—This was a very large growth of long standing that involved the entire right arm of a young man. The cell conditions before treatment, as revealed by biopsy, are shown in Fig. 57. Fig. 58 was made from biopsy material excised 4 days after administration of a single dose of 0.01 mgm. of polysaccharide. Externally, the sarcoma was black and blue because of hemorrhage and the gross appearance of the interior, once hard, white and glistening, was soft and blood-filled. Cells from tissue adjacent to these areas showed degeneration similar to that in mouse sarcomas.

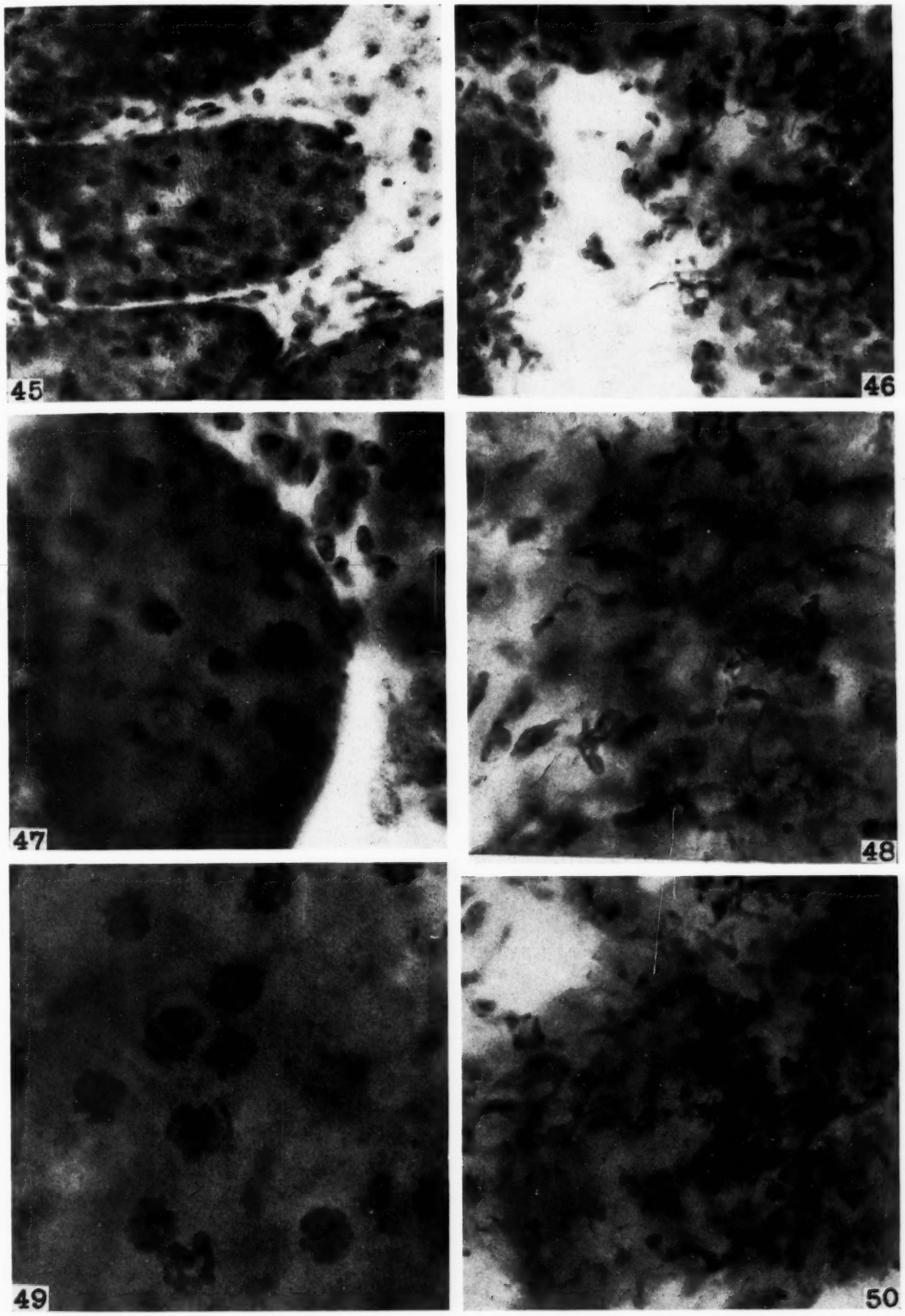
Chondrosarcoma.—The patient had several metastatic tumors growing in the nose and orbital fossa and was beyond further surgical aid. She was given a total of 0.033 mgm. of polysaccharide in 3 separate applications. Two biopsies were taken, at 4 days and 6 weeks, respectively, after treatment. The first posttreatment biopsy showed a part of the tumor to be extremely hemorrhagic. Cells from this region were in the anticipated stages of degeneration (Fig. 56).

Hodgkin's disease.—Another example of polysaccharide response appears in Fig. 60 taken from a neoplasm originally diagnosed as lymphosarcoma of the axillary nodes, but which was later determined to be Hodgkin's disease. The patient was given a total of 9 cc. or 0.09 mgm. of toxin, in three equal doses over a 2 day period; 3 days after cessation of treatment the sarcomatous nodes in the supraclavicular space were dissected out. In contrast to those removed before treatment (which had been firm and of the usual color) these were cyanotic, soft and necrotic. A comparison of the two figures taken from this material (Figs. 59 and 60) shows the extreme extrusion of chromatin threads after treatment, and degeneration of cell nuclei.

Lymphosarcoma.—This neoplasm was imbedded in the groin and was so indefinitely delimited that it was inadvisable to secure biopsy material for pretreatment examination. Therefore, no control figures are presented. The patient was given intravenously 4 cc. (0.05 mgm.) of polysaccharide, followed by the administration of 3 cc. (0.03 mgm.) by continuous intravenous drip; 6 days later, the tumor had become sufficiently shrunken and defined to enable the removal of what appeared to be the entire growth. Photographs of 2 different regions are presented in Fig. 61 and 62. Again there is noticeable filamentous extrusion of the chromatin substance from the nuclei, which are almost totally degenerative in the affected areas.

Polysaccharide Combined with X-Rays

Because of a clinical finding that the administration of polysaccharide apparently assisted in breaking down in a few cases the resistance of human tumors to irradiation, we made a few exploratory experiments concerning the effect on mouse sarcomas of combining polysaccharide injection with x-radiation. Mice with 7 day tumors were treated with 0.01 mgm. of polysaccharide, and 4½ hours later the tumors received a single irradiation of 2,500 r. Dual controls were maintained: (a) Animals with tumors treated with

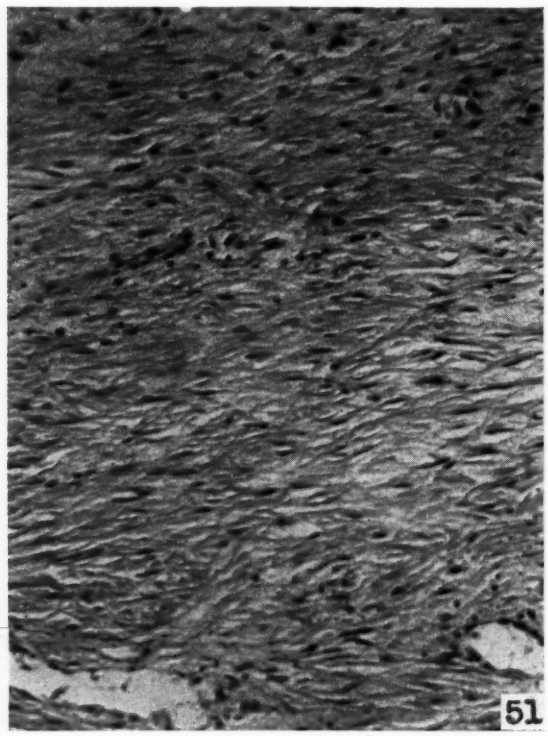


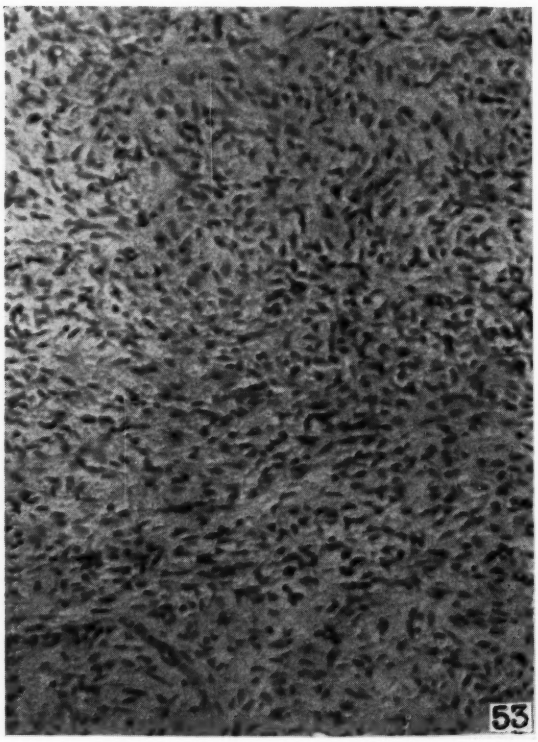
Photomicrographs of acetic orcein smears from normal body tissues of mice without tumors, treated with 0.1 mgm. of *S. marcescens* polysaccharide.

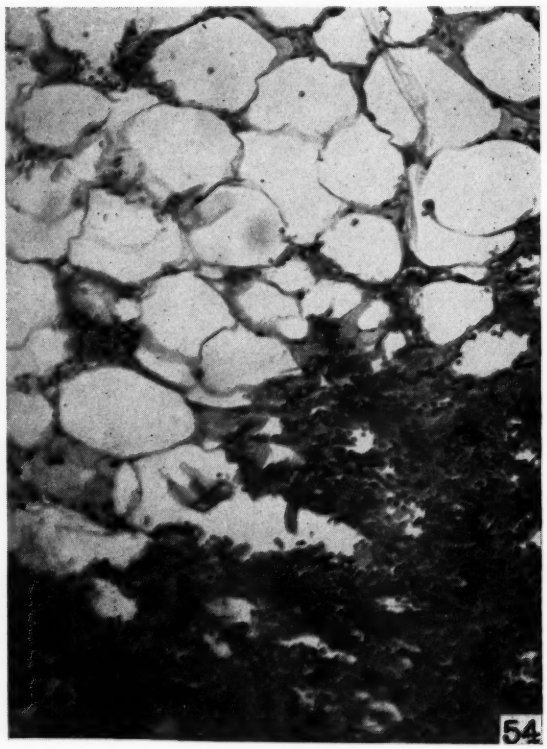
FIGS. 45 and 47.—Low and high power studies of intestinal epithelium of mouse, untreated.

FIGS. 46, 48, and 50.—Low and high power studies of intestinal epithelium of mouse, 6 hours after treatment.

FIG. 49.—Germ cells from mouse testis, undamaged, following administration of 0.1 mgm. of polysaccharide.







Photomicrographs from paraffin preparations of tumors in human patients, treated and untreated. Mag × 360.
FIG. 51.—Human chondrosarcoma, untreated.
FIG. 52.—Same, 4 days after treatment.
FIG. 53.—Fibrosarcoma, untreated.
FIG. 54.—Same, 4 days after treatment.

0.01 mgm. of polysaccharide; (b) animals with x-ray-treated tumors only (2,500 r in a single application). All x-rayed mice were placed beneath specially prepared lead shields, in which small openings were cut to allow the tumor to project beyond the shield, and the tumors were treated without removing the hair. The amount of x-ray administered was calculated[2] to be approximately equivalent to 500 r over bare skin.

Some animals were killed for microscopic examination of the tumors, but the remainder were maintained until the tumors were shed, or until their continued growth produced the death of the host. Grossly, about 25 per cent of the tumors responded to polysaccharide alone, as was expected. An almost identical number of degenerates was obtained in the group receiving only x-rays. The combined treatment, however, yielded as high as 75 per cent response, as indicated by total sloughing of the tumors, which showed that the effect was not merely an addition but an enhancement of their combined effects. Microscopic examination revealed that in animals killed at 24 and 72 hours after combined treatment, cellular response, too, was something more than the addition of the two types of damage.

It is well known that cells are most sensitive to x-rays at some stage of mitosis, probably metaphase. Ludford (11) reported that in tumors the mitotic effects of irradiation are greatest on those that are fastest growing. In sarcoma 37 no great change was produced microscopically at 6 hours when x-rays alone were employed; but at 24 hours there was a large amount of degenerating tissue, which apparently arose from chromatin fragmentation. Nevertheless, in only 25 per cent of the animals so treated were the tumors completely destroyed. When the two agents acted together only the expected polysaccharide effects were evident microscopically at 6 hours. However, at 24 hours a combined reaction (polysaccharide degenerates and filaments, plus fragmented chromatin strands) could be detected. The resulting masses of debris were often reduced to fine particles and fragments, as though the filamentous degenerates produced by polysaccharide had also been fragmented by the x-rays. A new phenomenon, moreover, appeared in resting cells. Nuclei of many of these were broken into rather large pieces, which still remained sufficiently in juxtaposition to render it possible to identify them as interkinetic nuclei. Such nuclei are shown in Figs. 21 and 22. The oval contours are lost, the nucleus is diminished in size, and cross-fragmentation of the entire nucleus has taken place. These changes did not appear in either of the control series. Since both agents act destructively on cells undergoing mitosis, we should not have been surprised had a combined single treatment produced no greater effect than either alone. The observations reported here are of a preliminary nature only, and much more experimentation must be done in order to determine how x-rays and polysaccharide in combination act to overcome the comparative resistance of the resting cell and also in what way minimum amounts of these two drastic agents can be combined for optimum effectiveness in destruction of tumor tissue.

[2]The author wishes to thank the staff of the Department of Radiology of the Lankenau Hospital for advice and technical assistance.

DISCUSSION

The assumption that tumor cells respond directly to the toxic effect of *S. marcescens* polysaccharide and that the resultant degeneration may be independent of or supplementary to the anoxia following breakdown of blood supply appears to be substantiated by the evidence presented. In the first place, it does not seem necessary that hemorrhage occur in order to obtain cellular degeneration, since at the time the initial swelling of the cell begins in response to polysaccharide there may be as yet no trace of hemorrhage within the tumor. Although sarcomas that are completely filled with blood at 6 hours post-treatment usually show a greater amount of necrotic tissue than do those that are only moderately hemorrhagic, nevertheless when nonhemorrhagic areas of tumors are selected for study, the cells are found to be reacting in a fashion identical with that observed in cells from blood-filled areas, except that the coagulation phenomenon is not present.

Care was taken to secure bits of tissue from both hemorrhagic and clear areas in order that microscopic comparisons could be made. In biopsy specimens of large human tumors, the areas of degenerating cells were usually located somewhere in the region of the hemorrhage. However, the presence of blood was not the exclusive criterion of cellular destruction, as was shown when a patient with Hodgkin's disease with multiple cervical lymph node enlargements was treated. Some of these nodes responded by severe hemorrhage, while others did not. Even so, both hemorrhagic and nonhemorrhagic nodes surgically removed after polysaccharide treatment showed cellular response, although it was most extensive in the hemorrhagic nodes.

Furthermore, following polysaccharide administration cell destruction in normal body tissues of

the mouse is not heralded by any marked hemorrhage of tissues; and in intestinal epithelium where damage was greatest, we could never detect macroscopic bloody areas marking the responsive regions, although many blood cells could be found among the degenerating tissues. Neither were there any hemorrhagic patches on liver or spleen following administration of an amount of polysaccharide 10 times as great as that required for tumor necrotization, although destruction of dividing cells in these organs was microscopically demonstrable.

Algire's studies (1) of the vascularization of sarcoma 37 and other tumors led him to observe that "an outstanding characteristic of the tumor cell is its capacity to elicit continually the growth of new capillary endothelium from the host," and Ludford (12) asserted that the vascular damage produced by colchicine in rapidly growing tumors is due to the fact that "the endothelial cells of newly formed capillaries are particularly sensitive to mitotic poisons." A study is now under way to determine, if possible, whether mouse tumor capillaries likewise break down because of polysaccharide destruction of rapidly proliferating endothelial cells. Frequently in rapidly growing tumors, cell multiplication proceeds with a speed with which capillary growth does not keep pace, and the blood travels not in formed capillaries but in spaces surrounded by closely aligned tumor cells. If these tumor cells break down under polysaccharide, hemorrhage naturally follows.

Bearing on these points is the example already mentioned of the stability of capillaries in a less rapidly dividing neoplasm (spontaneous rat carcinoma). In this instance it appeared clear that cellular degeneration was not due to the anoxia incident to deprivation of blood supply.

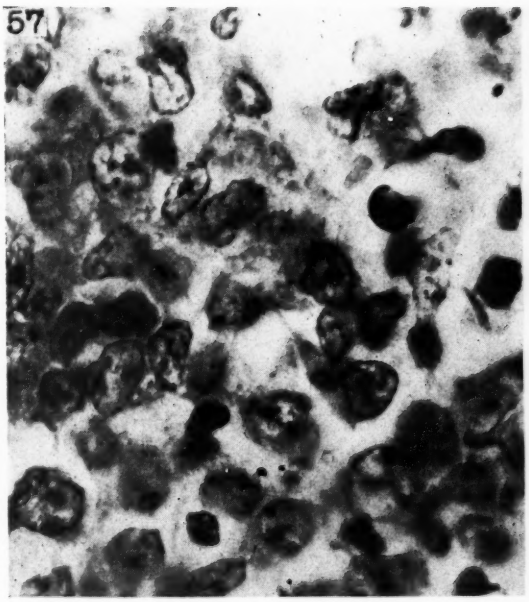
It can hardly be possible, however, that tumor destruction following polysaccharide treatment is entirely dependent on the direct toxicity to tumor cells; for it is obvious that where lack of oxygen and food supply have occurred through breakdown of the vascular system, there must be additional degeneration of the cells arising from these causes. Moreover, Algire (2) demonstrated by means of the mouse chamber that stasis is observed 3 to 4 hours after polysaccharide, and this may be an added factor in cell degeneration in the earlier stages of response. There is also the possibility that damage to cell walls may result in the leaking of plasma into the intercellular spaces several hours before the red cells come through.

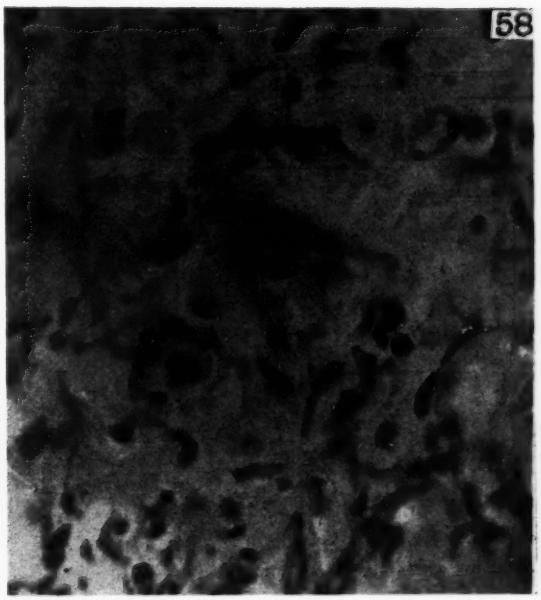
Furthermore, even when some of the tumor cells remain relatively healthy, coagulation of blood and formation of a scab may dispose of some of them by purely mechanical means. That is, the hardened mass of tissue breaks away from the skin and sloughs off, carrying with it some still viable cells mingled with the debris.

On the other hand, we have sometimes observed that tumors which were not sufficiently hemorrhagic to form a hardened mass of blood nevertheless were totally resorbed, indicating that the cells must have been destroyed. These were usually small tumors. In no case was the resorption of a large tumor observed, and this is probably correlated with the larger amount of resting and therefore less responsive tissue present in the older, larger, growths.

Although most of our evidence points to a relatively selective action of the polysaccharide as regards dividing cells, there is no way of determining whether some of the interkinetic cells may not also be affected. However, it is certain that after polysaccharide treatment, cells in mitotic division are destroyed, the mitotic process is suppressed, and the only undamaged nuclei are those of nondividing cells. As shown previously in this report, the first manifestation of polysaccharide effect is a swelling of the prophase nucleus, accompanied by blistering and other changes in the nuclear membrane. This is followed either by collapse of the nuclear membrane or extrusion of the nuclear contents. In the latter case, there is a seeming failure of the swollen nuclei to withstand the pressure of internal fluid, and the nuclear membrane gives way at some point, permitting the solid structures to be extruded. This is the most characteristic feature of nuclear response to polysaccharide and is seldom encountered during spontaneous degeneration. Our colleagues at the National Cancer Institute report that they have noted this type of nuclear degeneration occasionally in untreated tumors, though it is much less extensive than in treated ones, and usually appears in tumors found to be regressing under bacterial contamination.

The factor of spontaneous degeneration of these tumors renders difficult the cytological evaluation of response to chemotherapeutic agents generally; nevertheless in our experimental tumors it was possible to distinguish between induced and spontaneous necrosis with reasonable certainty. For one thing, cells degenerating spontaneously do not exhibit nuclear membrane changes so far as we have been able to observe, and though nuclei become shrunken and pycnotic, no differential degeneration of dividing cells is demonstrable. Nuclei of all stages shrink down into pycnotic spheres which are much smaller than polysaccharide-induced remnants and which show no





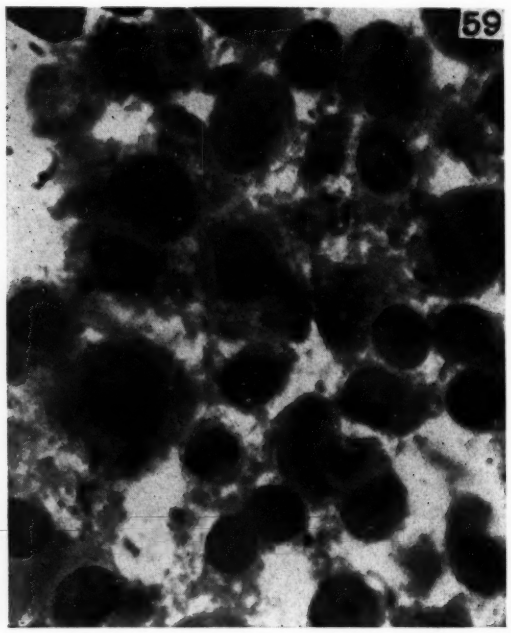
High-power studies (Zeiss 1.5 oil imm. lens) of tumors in human patients, control and treated tissue from biopsies taken before and after treatment. Mag. × 1,000 (approx.).

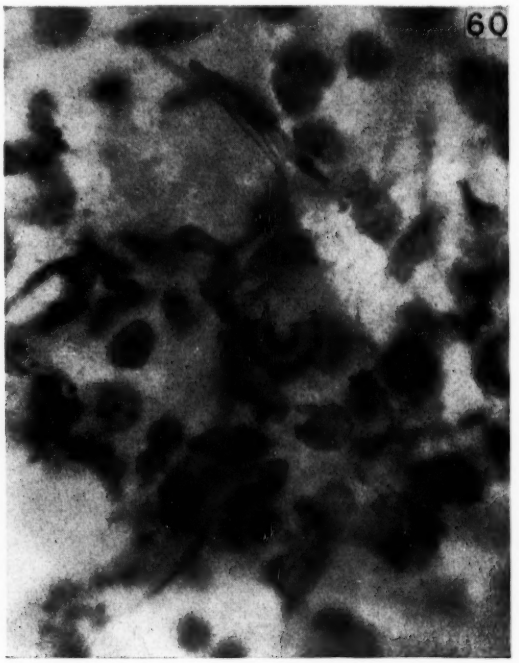
FIG. 55.—Human chondrosarcoma, untreated (acetic orcein smear).

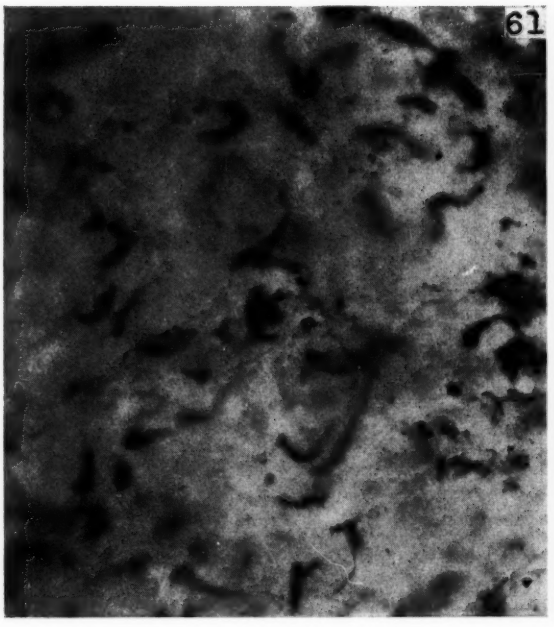
FIG. 56.—Same, 4 days after treatment (acetic orcein smear).

FIG. 57.—Human fibrosarcoma, untreated (acetic orcein smear).

FIG. 58.—Same, 4 days after treatment (acetic orcein smear).







Tumors in human patients, continued.

FIG. 59.—Untreated Hodgkin's lesion from cervical lymph node.

FIG. 60.—Same, 4 days after treatment.

FIGS. 61 and 62.—Human lymphosarcoma, 6 days after treatment, showing different regions of the neoplasm (hemorrhagic and non hemorrhagic).

trace of structure. Areas of spontaneous degeneration are usually filled with droplet-like globules of structureless, strongly orcein-positive substance or minute fragments that are likewise heavily stained and granular in appearance; that is, quite different in size and conformation from the components of cellular debris produced within a few hours after polysaccharide administration.

Following polysaccharide, no blistering or collapse of the nuclear membrane was observed in resting cells, even when they were inhibited from division.

The fact that at each progressive stage of nuclear degeneration following polysaccharide treatment there were present figures characteristic of each of the early stages, suggests that the process of morphologic change follows a rhythm, probably that of division, and that each nucleus runs a gamut of degenerative changes, depending upon the stage of division in which it existed on impact of the polysaccharide.

The assumption that the dividing cell is most responsive is bolstered by the observation that the cells not affected are those in resting stage and also by the reactions of nontumor tissue. Furthermore, the fact that there are no longer any anaphase or telophase figures present when the first of the affected nuclei reach the ultimate in degeneration indicates that mitosis was halted earlier. It should be borne in mind that in neoplasms with such a high mitotic index a large proportion of the cells are in some stage of mitosis at all times.

The reason carcinomas fail to respond as rapidly and vigorously to polysaccharide as do sarcomas is not clear and may of course reside in fundamental chemical differences between the cells of these two types of neoplasm. However, if our supposition is correct that dividing cells are more susceptible than resting cells, there may be a correlation between the restricted response of carcinomas and the small number of dividing cells present in any area of such tumors, as revealed by our controls.

It is uncertain whether cessation of mitosis during the 3 day period following treatment is due to polysaccharide suppression, or whether failure of cells to divide may result from loss of capillary connections. Algire's observations that capillaries are not re-established until the third day following polysaccharide damage may favor the second alternative. Our studies of young implants also bear on this point, since it was observed that wherever there are dividing cells they respond to polysaccharide, and that onset of division is dependent upon establishment of vascular connections.

Moreover, the same phenomena occur as were observed during the first 3 days following implantation and before capillary branching is elicited. That is, there are produced many enlarged nuclei with multiple nucleoli. This suggests that many of the cells continue to carry on the process of duplication of the chromatin through "endomitotic" activity (internal mitosis without nuclear or cell division) postulated in neoplasms by Biesele, Poyner and Painter (5). According to Biesele and his collaborators, such nuclei might theoretically arise in cells with an unusually high concentration of thymonucleoprotein "which might be explained on the basis . . . of synthesis over a long period of time." Such an opportunity for synthesis without chromatin repartition conceivably is afforded during the enforced interkinesis imposed either by polysaccharide inhibition or attendant anoxia.

Our studies of the growth pattern of sarcoma 37 have demonstrated that mitotic activity is not always localized at the periphery but that division centers are scattered throughout the tumor, wherever there is contact with vascular branches. The resultant random distribution of "resting" areas probably accounts for the fact that undamaged fragments of clear tissue, obviously capable of renewed growth, are often macroscopically observable at the periphery of the treated tumor, although the remainder of the growth may have become nothing more than a softened necrotic mass. In collaboration with other members of the Institute, studies are being made on the problem of destroying this persisting tissue through protection of animals against polysaccharide toxicity, breakdown of resistance to repeated treatments, and combination of polysaccharide with x-ray.

SUMMARY

S. marcesens polysaccharide produces nuclear damage to transplanted mouse sarcoma cells, separate from that arising through breakdown of the capillary system. Maximum destruction is attained at 6 hours, and only resting cells persist.

These cells are inhibited from division for 3 days following treatment. During this period they may persist morphologically unaltered, they may undergo some degeneration of cytoplasmic components, or they may become enormously swollen cells with huge nuclei and supernumerary nucleoli.

In a significant number of cases, no viable cells could be detected by our methods 3 days after treatment with a dose that killed 20 to 30 per cent of the mice. The same proportion of tumors was sloughed by animals maintained for survival studies. When polysaccharide was combined with x-rays this number was increased threefold.

Injection directly into the tumor did not inhibit growth.

Repeated treatments did not produce greater amounts of destruction, indicating that the tumor, or its host, had become resistant to the bacterial agent.

In primary neoplasms, including human sarcomas, the effects were similar to, but much less extensive than, those produced in mouse sarcomas.

ACKNOWLEDGMENTS

To Dr. M. J. Shear of the National Cancer Institute, I express great personal indebtedness for his help in initiating the problem, for his constant advice, and for generous sharing of research materials. I am grateful also to Dr. A. J. Donnelly, of our own staff, for pathological diagnoses; also to Miss Helen Robinson for help in the early stages of the study and assistance in matters pertaining to animal stocks. Finally, I wish to express gratitude to my own research assistants, and in the polysaccharide study particularly to Miss Priscilla Goodwin, for technical assistance of high order of excellence.

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Oral Carcinoma in a Monkey Colony

A Report of Two Additional Cases*

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A search of the literature discloses the extreme rarity of oral carcinomas in domesticated and captive wild animals. Only 15 cases of carcinoma of the tongue in domesticated animals (horse, cow, cat, dog) reported from various countries have come to our attention (14). The autopsy records of the 10,298 mammals and birds that died in the Philadelphia Zoological Garden during a period of about 30 years show that only one animal, a male bear, with a carcinoma of the tongue was found (11). As far as subhuman primates are concerned, the incidence of neoplastic diseases, according to Ratcliffe, has been and still is lower in the Philadelphia Zoological Garden than for any comparable group of mammals (12), and until 1947 no carcinomas of the tongue in monkeys were discovered (13). Nor have we seen any references to malignant neoplasms of the tongue in autopsy reports issued from other laboratories and zoological gardens in which monkeys have come to autopsy. The occurrence of 2 additional oral carcinomas in our laboratory colony of monkeys is of particular interest not only because of the previous occurrence of 3 carcinomas of the tongue in 2 other monkeys of the same colony (14), but also because the 2 cases to be reported involve different sexes and different divisions of the suborder *Pithecoidea*. Furthermore, we believe that the information available on these animals and on the environmental conditions under which the disease occurred is somewhat more detailed than is usually the case in studies of monkeys that have died in laboratories or menageries.

Case Reports

These 2 monkeys were members of a colony of catarrhine and platyrrhine monkeys kept by one of us (H. K.) for neurophysiological and behavioral investigations.

Monkey No. 1.—A male rhesus monkey (*Macaca mulatta*), purchased from an animal dealer in New York City, was received in the laboratory on October 7, 1939. Its age at the time of arrival was estimated to be approximately 8 years. It was kept continuously in the laboratory until its death on February 23, 1945. It is worth noting that throughout this period of observation, whenever the animal was found sitting quietly, its lower jaw drooped (Fig. 1). This behavior is not characteristic of normal rhesus monkeys. During January 1945 the monkey started losing weight and confined itself more and more to eating soft food only. Saliva drooled frequently from its mouth and an occasional twitching in the region of the left corner of the mouth was observed. An examination of the mouth under nembutal anesthesia, on February 22, 1945, revealed an ulcerating lesion at the tip of the tongue measuring about 1.8 cm. in diameter and extending transversely about 2.1 cm. at the under surface of the tongue. Biopsy disclosed a squamous cell carcinoma.

On February 23, 1945, the monkey was anesthetized with ether and then sacrificed by an incision into the heart. Eyes, ears, nose and throat were grossly normal. The tongue had an ulcerating lesion on the left tip extending from the papillary margin down to the floor of the mouth. The submaxillary gland as well as the muscular tissues and vessels on the left side seemed to be directly infiltrated by the tumor. A small isolated nodule was noted in the hard palate. The other submaxillary and sublingual glands as well as the main cervical lymph nodes of the upper part of the neck were involved to a greater or lesser degree by metastatic tumor. The thyroid was not identified. The lungs were normal except for anthracotic pigmentation. The mediastinum and its contents and the heart were normal. Lymph nodes were not involved. The thymus was not identified. The abdominal cavity seemed grossly normal, except for a few adhesions between the viscera, and the spleen was rather small and shrunken. The liver, gallbladder, extrabiliary ducts, pancreas, stomach and small bowel seemed normal. The

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large bowel was normal except for a cecal diverticulum. There was a moderate amount of retroperitoneal fat. The kidneys, adrenals, bladder, testes and prostate seemed grossly normal. The teeth were in good alignment and showed no visible caries with the exception of the maxillary third molar on the right side. Body weight: 7.7 kgm.

Microscopic examination of the primary lesion showed a squamous cell carcinoma irradiating deeply into the tongue musculature (Fig. 2). There were regions of anaplasia and regions where pearl formation was quite definite. Sections through the enlarged regional cervical nodes showed practically complete replacement of lymphoid tissue by metastatic squamous cell carcinoma (Fig. 3). The following tissues were examined and found to be negative: striated muscle, parotid gland, stomach, small bowel, gallbladder, kidney, adrenal, urinary bladder, pancreas, spleen and lung. The testes showed normal configuration and relationship of tubules and stroma. However, there was definitely less spermatogenesis apparent than would be expected in normal monkeys at the height of sexual potency. The liver showed no cirrhosis or cholangitis. There was extensive fatty infiltration of the liver cells.

Monkey No. 2.—A female squirrel monkey (*Saimiri sciurea*) was received from the Brookfield Zoological Garden (Illinois) on June 3, 1938, and kept under study in the laboratory until its death on March 1, 1944. It was estimated to be between 2 and 2½ years old at the time of its arrival. On November 6, 1943, a small reddish lesion which at first was not encrusted appeared at the right angle of the mouth. About two weeks later the animal was no longer able or willing to shell peanuts, but only in February 1944 was it apparent that the visible lesion had definitely increased in size with a progressive swelling of the right cheek. The animal was often seen rubbing its face against the boards of the cage and scratching its right cheek very gently. On March 1, 1944, the swelling extended from the angle of the mouth to the right ear and was about the size of half a walnut. During the preceding week the animal had not been able to consume any food except milk and milk-soaked bread. When an examination revealed a large ulcerating lesion of the right buccal mucosa emitting an offensive odor, the monkey was killed by means of ether. Body weight: 568 gm. Brain weight: 22 gm. Owing to other demands on the laboratory, no detailed postmortem examination could be made. All organs, including the tongue and the teeth, appeared grossly normal.

Microscopic examination of the lesion of the right buccal mucosa showed a typical squamous cell carcinoma (Fig. 4).

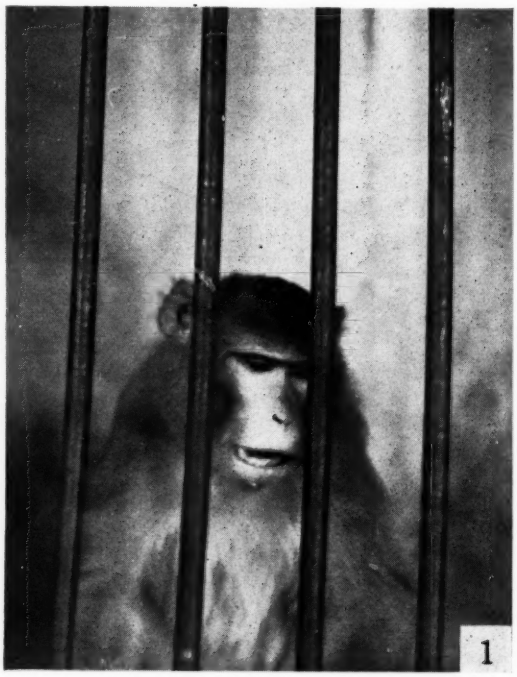

FIG. 1.—Monkey No. 1 (rhesus monkey) showing characteristic drooping of lower jaw.

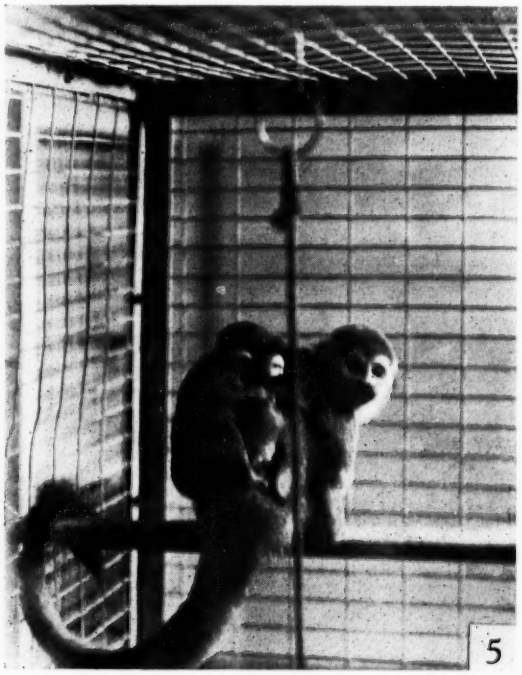

FIG. 5.—Monkey No. 2 (squirrel monkey) with baby

Conditions Associated with Occurrence of Cancers

Age factor.—At the time of death monkey No. 1 had lived approximately 5 years and 4 months and monkey No. 2, 5 years and 9 months under the conditions of this laboratory. If our estimate of the age on arrival is approximately correct, monkey No. 1 (rhesus) was about 13½ years old at the time of death and must, therefore, be considered aged in view of what is known about the life span of macaques. Published records provide no definite information on the duration of life in squirrel monkeys. Flower (3) discovered that a statement to the effect that a squirrel monkey lived as long as 9 years in the London Zoological Gardens was "founded on a clerical error." However, it is a fact and not a statement based on a clerical error that a female squirrel monkey lived 9 years and 9 months in our laboratory. This animal was apparently in good health and pregnant when an unknown disease suddenly brought about its death and, within a few days, the death of 3 other squirrel monkeys and of 3 night monkeys housed in the same room. Bacteriological examinations did not reveal the causative organisms. Since this particular female was at least 3 to 4 years old on arrival, we consider it likely that squirrel monkeys may reach an age of 15 to 20 years. It appears, therefore, that monkey No. 2 (squirrel) which was only about 8 years old at the time of death cannot be considered aged.

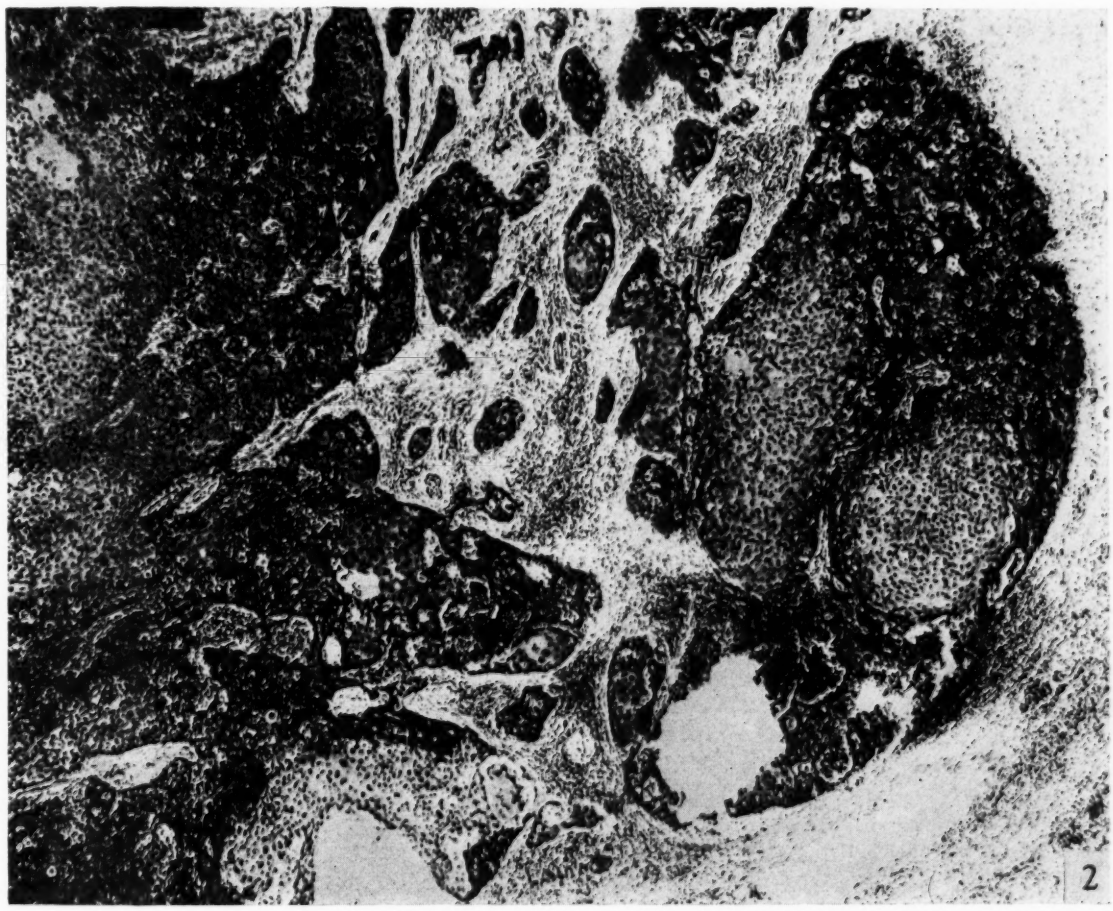

Fig. 2.—Photomicrograph of lesion of the tongue of monkey No. 1 showing typical squamous cell carcinoma with invasion of deeper lymphatics. Mag. × 62.

Hereditary factor.—Since monkey No. 1 was an Old World monkey and No. 2 a New World monkey, a common hereditary factor may be safely excluded.

Diet.—The diet used for the monkey colony, except for supplementing it with brewers' yeast, was the same as previously described (14) and appears to have been adequate even for South American monkeys. In view of the fact that in the course of a century only 4 births occurred among South American monkeys in the London Zoolog-

ical Gardens (17), it deserves emphasis that in our laboratory 9 such births occurred during a period of 5 years (1940-1945). Mention should be made of the fact that the daily supply of vegetables and fruits obtained for the animals always included some spoiled and decayed products. Only after cutting off the rotten parts and rinsing or washing the rest under running water was such food offered to the animals. However, there is no doubt that the removal of the visible rotten parts was not always complete and that the monkeys, throughout the years of their existence in the laboratory, often chewed and even consumed decomposed matter of plant origin. Although no hot food was ever offered, boiled food (potatoes) was at times part of the diet of the Old World monkeys.

When received in the laboratory, monkey No. 1 appeared to be somewhat emaciated and in rather poor health. It was listless and frequently had diarrhea, but with the care and diet given in the laboratory its general condition rapidly improved during the first 3 months. In fact, the gain in weight continued so that about 3 years later the monkey was fairly obese and started losing weight only during the last weeks of its life. Nevertheless, it did not always enjoy perfect health since it was at intervals subject to diarrhea and was now and then seen lying on the seat of its cage. Its dull expression and its slow movements when undisturbed made an observer easily forget that it was a powerful and aggressive animal ever ready to exhibit startling speed and agility in attacking any human being within reach and even succeeding in seriously injuring an experienced caretaker. As a rule, normal rhesus monkeys do not accept or eat meat offered to them although fully mature or old animals recently arrived from India may occasionally do so. In testing the food preferences of monkey No. 1 it was observed that the animal accepted and slowly ate pieces of sausages or boiled tongue. It seems likely that it had been forced at times to rely on animal products for food in its natural habitat, where it probably also ac-

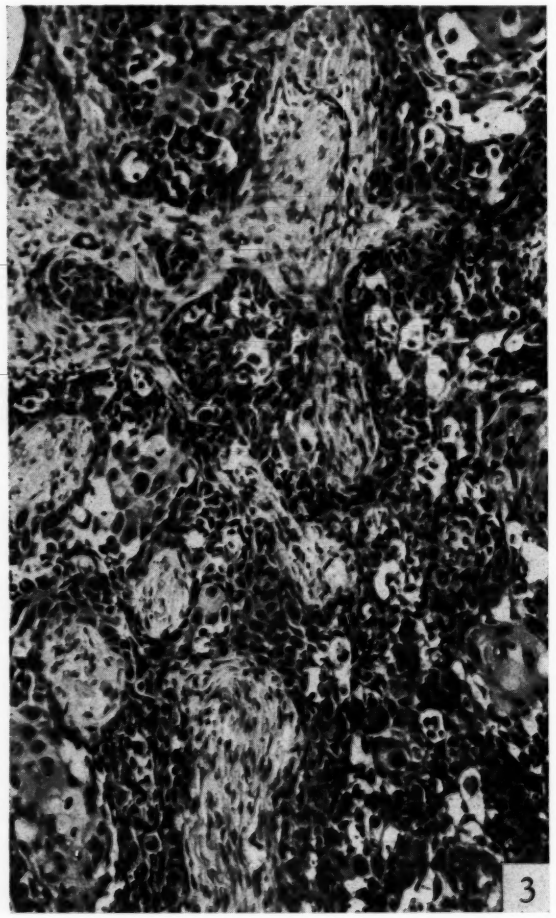

FIG. 3.—Photomicrograph of metastatic squamous cell carcinoma in cervical lymph node from primary lesion shown in Fig. 2. Mag. × 125.

FIG. 4.—Photomicrograph of primary lesion of the buccal muco a of monkey No. 2 showing typical infiltrating squamous cell carcinoma. Mag. × 77.

quired its extreme fear of snakes which extended to toy snakes and wavy lines drawn on cardboard. In connection with dietary habits it is of further interest that the animal when thirsty would drink a 10 per cent NaCl solution, but not a saturated aqueous solution of quinine sulfate (as done by some apparently healthy monkeys).

Monkey No. 2 appeared to be in excellent health from the very beginning of its life in the laboratory. Although squirrel monkeys in captivity readily consume numerous animal products, no meat was included in the diet provided for monkey No. 2. However, it was given a raw egg once a week and it supplemented this diet by eating the live cockroaches it caught every day. In view of the fact that all normal monkeys of the colony thrived under these conditions as indicated by general appearance, behavior, longevity, the occurrence of births, the growth curves and general condition of the offspring, it seems unlikely that the lesion at the right angle of the mouth in monkey No. 2 was the result of nutritional deficiency.

Brain lesions.—Since the previously reported carcinomas occurred in monkeys with brain lesions (14), it is of special interest that one of the two cases reported here had also undergone a brain operation. On January 24, 1941, monkey No. 1 underwent extirpation of the left temporal lobe including most of the amygdala, hippocampus and uncus. Anesthesia was induced by an intraperitoneal injection of nembutal. The Bovie high frequency current and blunt spatulae were used in connection with the operation. There was an uneventful recovery.

Hormonal and sex factors.—Throughout the years of its laboratory existence monkey No. 1 exhibited a red coloration, unaccompanied by swelling, of the "sexual skin," including the scrotal skin. There was only an occasional blanching, but at times there was also a reddening as intense as that seen in pregnant females of this species. Incidentally, the same intensive reddening may at the present time be observed in all aged or old male rhesus monkeys of our colony. In monkey No. 1, no overt sexual behavior was ever observed except masturbation during the week following the brain operation. It is known that the reddening and swelling of the sexual skin in the female rhesus monkey develops progressively during the follicular phase (1, 18, 19) and that the inhibitory effects of progesterone on the effects of estrogen on the sexual skin are particularly marked in juvenile monkeys (1). The response of the region of the sexual skin in normal male rhesus monkeys seems to be similar to that seen in females (16,19). Zuckerman (18) has considered the possibility that the activating agent of the sexual skin in male monkeys is not an estrogenic substance. It is of interest in this connection that Dr. F. C. Koch found 6 international units of androgens (1.22 units/kgm. of body weight) per 24 hours and no estrogens in capon assays on the urine of a sexually mature male rhesus monkey of our colony. However, there was no reddening of the sexual skin at the time the assays were made and red coloration developed only years later. It will be necessary to study the daily urinary excretion of androgens and estrogens in male rhesus monkeys exhibiting continuously a brilliant sexual skin.

In the case of monkey No. 2, the period of visible development of lesion and tumor fell into a period of gestation and lactation. The first signs of pregnancy became manifest 7 weeks before the appearance of the small lesion at the right angle of the mouth. Monkey No. 2 gave birth to a male baby on November 24, 1943 (Fig. 5). We have not seen any published records indicating that birth of a squirrel monkey has ever previously occurred in captivity. Nursing and maternal behavior as well as growth of the baby seemed to be normal in all respects. The infant was only about 14 weeks old when it became necessary to sacrifice the mother, but its development was such that it continued to thrive on solid food and milk.

Infectious and contagious factors.—The 2 monkeys were housed in different rooms of the same building. The cage in which monkey No. 1 was always kept alone stood in a room used as quarters for rhesus monkeys and a number of monkeys belonging to other species. Monkey No. 2 always occupied a cage together with the other squirrel monkeys of the colony. The same room contained cages housing night monkeys and rats. All animals were exposed to the same caretakers and investigators. There was no evidence of tuberculosis or other diseases in the colony during the years monkeys No. 1 and No. 2 lived in the laboratory.

Porphyrins.—In both monkeys the ulcerated portions of the carcinomas exhibited a striking red fluorescence when they were illuminated by light from a mercury vapor lamp passing through a Corning filter No. 5874. The fluorescence spectra of these tissues suggested the occurrence of free porphyrins. In monkey No. 1 the red fluorescence as well as a fluorescence spectrum characteristic of porphyrins were seen only in the cancer tissue of the tongue, but not in the metastatic tumor. In monkey No. 2 it was the surface of the lesion involving the right buccal mucosa which exhibited the most brilliant red fluorescence. When an examination of a cross section through the right cheek was made, it was apparent that the red

fluorescence became less intense with increasing distance from the ulcerated surface and that it finally changed into a violet and whitish-violet fluorescence. The lesion at the right angle of the mouth, even after removal of its encrustation, showed a deep red fluorescence. In both monkeys, small portions of the tissues exhibiting red fluorescence were removed for spectrochemical investigation. An extraction of the tissues provided evidence, chiefly based on solubilities and the measurements of fluorescence spectra in different solvents, that the cancer tissue from monkey No. 1 contained deuteroporphyrin and that from No. 2, protoporphyrin. In addition, traces of other ether-soluble porphyrins were present, but the amounts were not sufficient for identification (probably protoporphyrin in the case of monkey No. 1 and coproporphyrin in No. 2). The fluorescence of cancer tissues has engaged the attention of several investigators (2, 4, 5, 7-10, 15). Although porphyrins have been held responsible for the red fluorescence occasionally seen in cancers, it seems that such porphyrins have not been isolated and identified by extracting the cancerous tissues. In examining rat sarcomas, Policard (10) observed that the necrotic center of the inoculation tumor showed an intense red fluorescence surrounded by a narrow violet zone which was again encircled by a peripheral zone showing white fluorescence. Körbler (7, 8) described and photographed the red fluorescence of human ulcerated carcinomas. He was not able, and Policard did not even try, to isolate the porphyrin supposedly responsible for the red fluorescence. Both investigators believed hematoporphyrin to be the agent responsible for the effects observed, but we know at the present time that it is not one of the naturally occurring porphyrins. In considering the origin of the porphyrins found in the ulcerated cancer tissues of monkeys No. 1 and 2, it seems safe to consider, first of all, such factors as bacterial action and the degradation of blood and food remnants. We have previously reported that an intense red fluorescence may be observed in the necrotic wounds of monkeys and that various porphyrins may be extracted from necrotic tissue (6). Neither the extractions of portions of the metastatic tumor in monkey No. 1 nor the spectroscopic examination of the non-ulcerated portions of the tumor in monkey No. 2 provided any definite evidence for the occurrence of porphyrins. It is worth mentioning that the tartar found on the molars of monkey No. 1 exhibited a red fluorescence and a fluorescence spectrum with an emission band at about 620 mμ. Since we have made similar observations on many normal monkeys it is doubtful whether any special significance can be attached to the finding. Extractions of such deposits always furnish one or several ether-soluble porphyrins.

Various environmental factors.—The conditions prevailing in the laboratory as to temperature, illumination, housing, and food utensils have been previously described (14). The animals were under close observation, but no injuries of the tongue were ever noticed. The food never included such items as oats and barley. With exception of the anesthetics mentioned, no drugs were ever administered. Other animals present at various times in the monkey quarters of the laboratory were cockroaches, mice, cats and human beings. Since the laboratory building was once used for work in chemistry, it was considered desirable to check on the presence of radioactivity. Dr. Nickson from the Argonne National Laboratory reported that the spot checks around monkey cages, sinks, door knobs, floors, walls and cabinets failed to reveal any alpha, beta or gamma radioactivity exceeding 100 alpha counts per minute per 100 sq. cm., or 0.1 mr. per hour at 1 inch.

SUMMARY

Two squamous cell carcinomas occurred in 2 monkeys of a laboratory colony in which 3 squamous cell carcinomas of the tongue in 2 other monkeys had previously been observed. A squamous cell carcinoma of the tongue with local tumor metastases occurred in a male rhesus monkey and a squamous cell carcinoma of the right cheek in a female squirrel monkey. The period of life in the laboratory was approximately 5½ years for both monkeys. The South American squirrel monkey was not considered aged at the time of death. The male rhesus monkey, an animal with an experimental brain lesion of long standing, exhibited an almost continuous reddening of the "sexual skin." In the squirrel monkey the period of the visible development of the tumor fell into a period of gestation and lactation. In both monkeys the ulcerated portions of the carcinomas showed a striking red fluorescence. The porphyrins isolated from the ulcerated tissues were deuteroporphyrin and protoporphyrin. The occurrence of the disease could not be definitely related to any of the etiological factors supposedly responsible for oral carcinomas in man. The fact that of the 30 monkeys in the stock colony of the laboratory 4 have been found to have oral carcinoma (a gross incidence of 13 per cent of this population) and that monkeys belonging to species as different as *Macaca mulatta* and *Saimiri sciurea* have been among the affected animals strongly suggests that an extraneous factor may have played an etio-

logical role although there is no indication as yet what this factor may be.

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Antibody Response to Tumor Inoculation in Mice

With Special Reference to Partial Antibodies*

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INTRODUCTION

The present writer (8–10) has advanced the theory that the genes determining the fate of transplanted tissues are identical with those determining the presence of iso-antigenic differences. Such differences are most easily studied in erythrocytes. Strong's A strain happens to possess two iso-antigens that are shared by the erythrocytes and a number of fixed tissues, including all neoplastic tissues studied so far. These antigens have been called antigens I and II, the latter being the more potent. The best antibody producers found to date have been members of Little's C57 black strain.

A spindle cell sarcoma and a myeloblastic leukemia arising in the A strain have been tested on hybrids between these two strains. In each case it appeared that 2 dominant genes were essential for growth, one of which appeared to be identical with the gene for antigen II. Sera for the detection of antigen I are difficult to obtain and were not available for the genetical investigations. Mice in which the tumors had regressed produced iso-agglutinins; in most cases these could be identified as "anti-II," but certain sera contained an additional agglutinin that reacted with the cells of mice of the CBA strain, which shares antigen I with the A strain but lacks II. It seems likely, therefore that both neoplasms contain antigens I and II (9). Agglutinins may also be produced by inoculation of blood or normal tissues, neoplastic tissues giving the better response, partly perhaps because of their greater proliferative power, but possibly because they contain more of the pertinent antigens than normal tissues (9, 10).

The results summarized in the preceeding paragraph were obtained with animals that had been inoculated once. The response to hyper-immunization was studied principally in connection with leukemia. Here it appeared that following two or more injections, the hemagglutinins disappeared. However, sera that were apparently free from agglutinins could protect mice of the A strain against inoculation with leukemic cells, as could those from which the agglutinins had been removed by absorption. It was deduced that mice produced two sorts of antibody, agglutinins and protective antibodies. The latter could be partially absorbed by red cells but far less readily than by leukemic cells, indeed the absorption by the former was difficult to detect. However, the agglutinins are far more easily absorbed by neoplastic cells than by erythrocytes and the conclusion was reached that whilst the two antibodies were qualitatively distinct, they were both directed against the same antigens (10).

At this point the investigations were interrupted by the war. In the meantime, important advances have been made in the study of iso-immunization to the rhesus antigens in man. Here again it appears that two qualitatively distinct antibodies may be formed, ordinary agglutinins and partial or blocking antibodies (11, 13). The latter need special methods for detection *in vitro*, reference to which will be made below, but they appear to be of greater functional significance and are sometimes referred to as the mature type of Rh antibody (3, 15). This situation shows interesting analogies to that just described for mice and we, therefore, decided to see whether such antibodies could be detected in this species.

MATERIALS AND METHODS

The Roscoe B. Jackson Memorial Laboratory had two A strain tumors available, known as 15091a and C1300. The former has been described as a spindle cell mammary carcinoma. It is a pleomorphic anaplastic growth with fairly numerous giant cells. The latter is a round cell tumor, possibly a neuroblastoma. Of the two, 15091a is much more virulent for heterologous pure strains, causing a high mortality in many of them. In the

*All the experiments reported here were performed at the Roscoe B. Jackson Memorial Laboratory. The work was supported by the National Institute of Health and by The Jane Coffin Childs Memorial Fund for Medical Research.

C57 black mice it seldom causes death but far larger masses are usually produced than is the case with C1300. They were inoculated in the ordinary way by trocar and canula.

Previous experience has shown that the agglutination tests must be performed in tubes and that in reading, care must be taken to avoid too much force in breaking up the cellular deposit. The author (7, 9) has found the following method suitable: About 0.05 cc. of serum dilution and 1 per cent cell suspensions are set up in dwarf test tubes and incubated for an hour or more at 37°C. The tubes are then centrifuged for 2 minutes at about 700 r.p.m. (it is important that spinning should not be too violent or too long). The readings are then made by *gently* pumping the supernatant back and forth with a capillary pipette. In the absence of agglutination the cells come away as an even cloud, sometimes leaving a small wisp of cells attached to the tube. After pumping 4 times most of the deposit should be resuspended If there is feeble agglutination, the deposit may be easily resuspended but has a granular appearance. As the strength of the reaction increases, the deposit breaks up into lumps of increasing size and often comes off as a solid pellet. There is also a tendency for agglutinated cells to stick to the glass, and this in itself is often a helpful criterion. There is only one real source of confusion in microscopical examination. In controls one may often see a few large clumps of cells. If the slide is gently tilted these will break up, the cells coming away singly. If they come away in small clumps there is some agglutination.

When doing tests where the final concentration of serum or plasma is relatively low, it is not necessary to wash the cells. If the final concentration of serum or plasma is high (say 50 per cent or over) washing is essential. It has been found most satisfactory to perform the washing with the 1 per cent suspensions. These are centrifuged at about 700 r.p.m. Two such washings are sufficient although 3 have sometimes been used. If the cells are not washed but suspended in pure serum, the whole mixture may clot, or partial clotting may occur, closely mimicking agglutination.

For the detection of partial antibodies in man the following technics have been used: the blocking test (11, 13), the conglutinin test of Wiener (14), the anti-globulin test of Coombs, Mourant and Race (2), the open slide (4) and albumin tests of Diamond and his co-workers (6). All of these have been tested. The last two seem inapplicable to mouse antisera. Human albumin was used and found to be lytic for mouse cells in saline; the lysis was inhibited by serum, but it did not bring about agglutination by blocking antibodies. The Coombs test gave some suggestive results and will have to be retested with more potent sera.

The technic of the blocking test will be described below. Wiener's conglutinin test depends upon the performance of all operations in compatible serum or plasma. This has been used successfully although the results are slightly different from those obtained with human sera. In this case it has not been found essential to avoid the slightest dilution of serum. When testing some sera the cells have been suspended in saline and all antibody dilutions done in compatible serum. This gives a final concentration of serum of 50 per cent. The most satisfactory system for general use has been with the serum dilutions as before but with the cells suspended in 50 per cent serum, giving a final concentration of 75 per cent serum. Where the standard method has been used, it is referred to simply as "saline." Except where stated to the contrary, pooled sera from 6 mice were titrated.

RESULTS

The Detection of Antibodies in Normal Serum

Up to the present it has been generally agreed that mice do not possess natural iso-agglutinins. It was, therefore, surprising to find that sera of C57 blacks would often cause agglutination of A strain erythrocytes.[1] At first it was thought that this might be non-specific agglutination due to some undetected technical error. This is not the case since C57 black cells are not agglutinated. Originally the discovery was made with pooled sera from 6 or more mice. Later it was found that about 20 per cent of blacks gave some agglutination. Usually the reactions were feeble and could only be detected if the cells were suspended in the sera. However, in one pool strong agglutination was found. The antibody reacted with the cells of strains A, C3H and dba, but not with those of Bagg albino C, or the C57 blacks.

At the present time it is not possible to say what stimulus elicits these iso-antibodies. They have been found in both sexes, but it is possible that fetuses containing the pertinent antigens may sometimes stimulate their formation. This aspect of the problem would probably repay investigation.

The natural iso-antibodies can easily be absorbed, but it has been found more convenient to use the sera of strain A or C3H animals as a vehicle, and this has been done in the experiments to be reported below.

[1] This has been found to be true of Swiss mice also.

The Antibody Response to Blood

It is well known that no two tumors are exactly alike and that a given tumor may undergo variation. Of the normal tissues, blood has many advantages and a few preliminary experiments were therefore performed in order to see if the results were of the same kind as those obtained previously. Citrated whole blood was inoculated intraperitoneally, allowing for the dilution about 0.25 cc. were given at each inoculation. In the first experiment (*see* Table I) the inoculations were given at weekly intervals. The mice were bled after the first, second and fourth inoculations. In the second experiment the mice were injected twice a week until about 1.0 cc. had been given, when they were bled. They were rested for three weeks and then given two inoculations of 0.5 cc. within a week. The objective of the second experiment was to keep the mice constantly flooded with antigen as probably occurs with a tumor that proliferates and then regresses.

Table I: The Antibody Response to Whole Blood

Dose of blood given, cc.	No. of days since last inoculation	Technic*	Dilution of serum 2	4	8	16	32	64	128	256	512	Remarks
0.25	7	Saline	+++	++	±	tr	−	−	−	−	−	Dosage at weekly intervals
		75% serum	a.c.	+++	+++	++	−	−	−	−	−	
0.5	7	Saline	+	−	−	−	−	−	−	−	−	
		75% serum	+±	+±	−	−	−	−	−	−	−	
1.0	7	Saline	+++	+++	c	c	c	a.c.	a.c.	+	−	
		75% serum	+++	a.c.	c	a.c.	a.c.	a.c.	a.c.	tr	−	
1.0	10	Saline	c	+++	++	tr	−	−	−	−	−	Dosage twice weekly
		75% serum	c	a.c.	+++	+	−	−	−	−	−	
2.0	10	Saline	++	a.c.	c	+++	++	+	tr	−	−	
		75% serum	++	a.c.	a.c.	a.c.	+++	+++	++	+	±	

*The meaning of the terms in this column are explained in the text.
c = complete agglutination; a.c., almost complete agglutination; tr, trace, etc.

The first series is perhaps the most instructive. It will be seen that following the second 0.25 cc. there is an apparent drop in the titer of antibodies, to be followed by a rise in titer after 1.0 cc. had been given. Comparison with the second series shows that the response is less good when 1.0 cc. is given in concentrated dosage over 2 weeks than when the course is spread over a month.

Normal serum did not enhance agglutination to any great extent in these experiments. It will be noticed that there is a suggestion of a pro-zone with the hyper-immune sera.

The Response to Tumor C1300

As is the case with blood, the spacing of the inoculations has an influence on the result. A short, intensive course of inoculations gave a lower titer than when they were spread out (see last titer, Table II). As a rule it is best to allow about 2 weeks to elapse following the first inoculation and give the subsequent ones whenever the tumor is ready to transfer. Probably about 10 day intervals are the best, but one can get almost the same result if the animals are rested a month or more and then given a booster inoculation.

The first four sera shown in Table II were all from the same group of animals. This is the type of response that may be taken as typical. Here again we see an apparent disappearance of agglutinins following a second inoculation. However, in this case titration in serum shows some agglutination with complete inhibition in the pro-zone. Following further inoculations there is a reappearance of agglutination in saline but the inhibition zone remains. This is almost invariably 1 tube shorter in serum. Further inoculations raise the titer and the inhibition zone tends to become shorter. In one group of animals it had disappeared after 6 inoculations. This is by no means always the case, however. An inhibition zone up to about $\frac{1}{8}$ usually remains. Increasing the number of inoculations still further may sometimes cause an apparent drop in titer.

The fifth titration shows the effects of prolonging the interval between inoculation and bleeding. In that illustrated in Table II all agglutinins active in saline have disappeared, whilst in serum there is a fair titer with a marked inhibition zone. In another case there were apparently no antibodies at 21 days. However, this particular serum gave a strongly positive blocking test.

These experiments indicate that there are 3 types of antibody produced: ordinary agglutinins, agglutinins needing normal serum for their activity, and blocking antibodies.

The Response to Tumor 15091a

This tumor has been studied in C57 brown as well as black mice. The latter are the more resistant but even here the response to primary inoculation is variable. The animals were usually inoculated

in groups of 12. Sometimes 2 or 3 of these died from the tumor. Thereafter 2 or 3 more groups were inoculated without a single death and with complete regression in about 2 weeks. This has been the common experience in recent months. The response to primary inoculation has been studied in the C57 blacks only. If there are persistent growths, one gets a serum with a marked inhibition zone as shown in the first entry serum in Table III. Following hyperimmunization in C57 blacks one does not get an apparent disappearance of antibodies, as occurred with the other two antigens. If the inoculations are given at very short intervals, one may get an inhibition zone similar to that occurring with C1300 (fifth serum, Table III). If they are spaced out further one seldom gets a pro-zone at all. If there is one, there is only partial inhibition of agglutination.

TABLE II: THE RESPONSE OF C57 BLACKS TO TUMOR C1300

No. of injections	Day of bleeding	Titred in	2	4	8	16	32	64	128	256	512	1024	Notes*
1	14	Saline	c	a.c.	+++	tr	−	−	−	−	−	−	First four titers, same group of animals
		75% serum	c	c	+++	+++	tr	−	−	−	−	−	
2	10	Saline	−	−	−	−	−	−	−	−	−	−	
		75% serum	−	−	−	−	tr	±	±	tr	tr	tr	
4	10	Saline	−	−	−	−	++	+++	±	±	tr	−	
		75% serum	−	−	−	++	+++	+++	+++	a.c.	+++	±	
4	23	Saline	−	−	−	−	−	−	−	−	−	−	
		75% serum	−	−	++	a.c.	c	+++	++	+	tr	−	
4	10	Saline	+++	a.c.	+++	++	−	−	−	−	−	−	Eighteen days between 1st and 2nd inoculation. Last 3 at 3 day intervals.
		75% serum	c	a.c.	+++	++	+	−	−	−	−	−	

One black mouse which was apparently dying of a large fungating growth, had blocking antibodies only. Dr. Snell was kind enough to give me some mice that had been rendered artificially susceptible by being inoculated with a lyophilized preparation of 15091a prior to the living tumor (12). These also were obviously *in extremis*. Their pooled sera gave the highest titer seen to date. It was over 16,000 in saline but only 4,096 in serum. This is an unusual finding.

It will be noticed that the ordinary agglutinins persist longer following inoculation with this growth than they do with C1300 (fourth serum, Table III).

Hyperimmunized C57 brown mice always give a pro-zone with this tumor. This was especially pronounced with serum of 2 animals with persistent growths. Two similar mice were tested individually. One had a smaller pro-zone than those shown in the table, with a titer of more than 4,096. The other showed blocking antibodies only.

Antibodies Needing Enhancement With Normal Serum

As can be seen from Table I to III, in most cases agglutination is inhanced by normal serum. Two cases in which it is essential have already been mentioned. Similar antibodies have been observed in the serum of animals that had recieved a slow-growing mammary carcinoma from the C3H strain. A more interesting example is given by animals that had been inoculated with doses of 5 mgm. of lyophilized 15091a. They showed a titer of 16 in serum but no agglutination in saline. As has been mentioned previously, Snell has shown that this treatment renders the animals highly susceptible to the living growth. Whilst the antibody response is feeble, it is interesting to note that it has not been completely inhibited.

Plasma may be used instead of serum, but it is awkward to store and cannot be used for experiments on the effects of heat. The enhancing factor (or factors) is thermostable, appearing to be undamaged by exposure to 60°C. for 30 minutes. As a rule the serum is used almost as soon as it is available. One sample that had been stored in the frozen state for a month did not appear quite so good as a fresh sample. It is perfectly safe to lay in a week's supply at a time.

The amount of serum needed appears to depend upon the concentration of antibody. With natural antibodies one may get agglutination with 70 per cent. With feeble antigens such as the C3H carcinoma, the titer will be appreciably lower in 50 per cent serum than in 75 per cent serum. With some of the anti-C1300 sera, one may get some agglutination with as little as 10 per cent. It is obvious that the situation is different from that found by Wiener with human serum where it is essential to avoid any dilution. Furthermore, mouse serum will not cause agglutination with true blocking antibodies.

The Detection of Blocking Antibodies

Blocking tests were first performed by Wiener and Race (11, 13) in connection with Rh sensitization, although Coca and Kelley had described essentially the same phenomenon in 1921 (1).

There are various possible modifications in the technic. In the present series of experiments two methods of titrating the sera were tried. In the first the test sera were diluted as in an ordinary

titration, incubated with red cells and a given quantity of agglutinin added to each tube. In the second case, undiluted immune sera were incubated together with red cells and varying quantities of agglutinin added. Examples of both of these are shown in Tables IV and V. Neither are ideal for titrating sera, although the latter appears preferable.

The first method has certain theoretical interest. The occurrence of a pro-zone suggests certain analogies with the inhibition due to antibody excess such as occurs with certain precipitating antisera from horses. It is difficult to check this with this is done the pro-zone is invariably reduced, the reduction being greater with increasing amounts of added antibody, as is clearly shown in Table IV. This is the opposite of what would occur if the inhibition were due to antibody excess.

TABLE V: BLOCKING TEST

Blocking serum undiluted. Agglutinin varied.

Cells incubated in:	Dilution of Agglutinin Added 2	4	8	16	32
Saline	++	++	+	+	−
Normal A serum	+++	+++	++	++	+
Blocking serum	−	−	−	−	−

The blocking serum used here was anti-C15091a from an animal with a tumor. There was not enough serum for further tests.

TABLE III: THE RESPONSE TO TUMOR 15091a

No. of inoculations	Day of bleeding	Titred in	Dilution of serum 2	4	8	16	32	64	128	256	512	1024	2048	4096	Notes
(A) C57 BLACK															
1	51	Saline	−	−	−	−	++	+++	−	−	−	−	−	−	Delayedregression.
		75% serum	−	−	−	+	++	+++	++	+	−	−	−	−	1 mouse subsequently died.
1	15	Saline	c	c	a.c.	+++	+	±	tr	−	−	−	−	−	
		75% serum	c	c	+++	+++	++	+	tr	−	−	−	−	−	
3	11	Saline	c	c	c	c	c	c	+++	+++	++	−	−	−	
		75% serum	±	c	c	c	c	c	a.c.	+++	++	−	−	−	
4	52	Saline	+++	++	++	++	++	±	−	−	−	−	−	−	
		75% serum	a.c.	a.c.	a.c.	+++	+++	+++	+++	++	−	−	−	−	
4	10	Saline	−	−	−	−	−	+++	+++	++±	++±	−	−	−	15 days between 1st and 2nd inoculation. Others at 3 day intervals
		75% serum	−	−	−	−	−	+++	a.c.	a.c.	c	+++	+	−	
1		Saline	c	c	c	c	c	c	c	c	c	c	c	c	Animals with huge growths. See text
		75% serum	c	c	c	c	c	c	c	c	c	+++	++	+	
(B) C57 BROWN															
3	12	Saline	−	−	−	−	−	−	+	±	±	tr	−	−	Tumors regressed.
		75% serum	−	−	−	−	−	++	+++	a.c.	c	a.c.	Not tested		
3	12	Saline	−	−	−	−	−	−	−	−	++	+++	Not tested		Large tumors.
		75% serum	−	−	−	−	−	−	−	+	++	a.c.	Not tested		Serum from 2 mice pooled.
4	21	75% serum	−	−	−	−	−	−	+	+++	+++	+++	+++	++±	Large tumor. Individual tested.
3	33	Saline	−	−	−	−	−	−	−	−	−	−	−	−	Large tumor.
		75% serum	−	−	−	−	−	−	−	−	−	−	−	−	Positive blocking test. 1 individual tested. See Table V.

excess antigen, since with very heavy cell suspensions the results are difficult to read. With suspensions up to 10 per cent the pro-zone did not appear to be shortened nor could this be done by adding light suspensions of malignant cells. However, in both these cases it is possible that insufficient antigen was added. Therefore, it seemed easier to approach the matter from another angle and see the effect of adding excess antibody. If

TABLE IV: BLOCKING TEST

Blocking serum diluted.
Constant amount of agglutinin added.

Agglutinin added	Dilution of Blocking Serum 1	2	4	8	16	32	64	128	256
Nil	−	−	−	−	−	−	±	±	±
Anti-C1300.									
4 units	−	−	−	±	+	c	c	Not read	
Anti-15091a.									
2 units	−	−	−	?	±	+++	+++	"	"

Controls: Saline + anti-C1300 (4 units) +++
Saline + anti-15091a (2 units) +

THE EFFECT OF STORAGE ON SERA

It had been observed previously that the isoagglutinins were largely destroyed by exposure to 60°C. for about 30 minutes. This observation has been confirmed with certain sera, but with others there was very little reduction in titer after such treatment. The other two types of antibody appear to be more thermostable. The sera were stored frozen solid, except in one case (the second serum in Table VI) which was left in the ordinary chamber of the ice box for 3 days. Apart from the fact that the agglutinins do not keep well, the behavior of any given serum is largely unpredictable. Sometimes there appears to be simple deterioration, but with many of the more powerful hyper-immune sera there is a gradual transformation to a blocking type of antibody. In some cases this is partially reversible by heat as shown with the first serum in Table VI. It will also be seen that the enhancing action of serum has become more noticeable. The second serum had kept

well until accidentally left at a higher temperature than usual. The last shows the gradual development of a pro-zone. It is of interest to note that Wiener (13) detected the formation of blocking antibodies on storage; in fact, he first noticed them in stored sera.

From many points of view the behavior of these sera is very inconvenient, but as will be shown below, it may throw light on the significance of the various forms of antibody *in vivo*.

DISCUSSION

It is of interest to compare the situation that obtains here with that occurring with Rh immunization in man. When one considers the taxonomic gulf that separates the two species, the results are surprisingly similar and might well be more so if the dosages given could be made at all comparable. In both cases it appears that a mixture of antibodies is formed, the type of result one obtains on titrating the sera depending upon their relative proportions. A pro-zone of complete inhibition is apparently much more common in mice than in man. This could be due to differences in dosage. The largest pro-zone was seen in a C57 brown animal with a large growth that had persisted for many weeks (Table III). It is difficult to believe that a situation at all comparable has ever occurred in man.

The present writer has been informed by more than one worker in the field that attempts to produce Rh antibodies by the inoculation of volunteers is apt to be disappointing as the titer of the complete antibodies may fall during immunization. This occurs in mice, but here they return again with sufficient dosage. In the experiments reported here this was about equal to the animals' blood volume.

The physical conditions under which cells sensitized with partial antibodies can be made to agglutinate are undoubtedly different in the two species. Nevertheless, in both it appears that these antibodies are not homogeneous. Coombs, Mourant and Race (2) found sera which gave negative results with the blocking test and with Wiener's conglutinin test, but was positive with their antiglobulin test. Similarly, Diamond and Abelson (5) found that about 7 per cent of sera were negative both to direct agglutination and the blocking tests. The addition of the slide test enabled the presence of antibodies to be detected in nearly 100 per cent of cases as does the albumin technic (6). These authors have shown that sera showing no pro-zone and apparently weak agglutinins may have high titers of partial antibodies (6). It is not clear why some partial antibodies give a strong blocking effect and a pro-zone, whilst others do not. The precise type of titer one gets must be the resultant of the different proportions of all of them. It seems likely that the apparent drop in titer observed in the course of immunization with blood and with C1300 (see Tables I and II) is due to the presence of partial antibodies.

A further complication is introduced by the fact that we are not dealing with pure antigens. The two tumors used here contain at least two isoantigens in common, and it is interesting to note that Dr. Snell has found that in certain crosses C1300 gives two gene ratios. It is striking that all of four A strain tumors (2 studied here and 2 in England) have appeared to contain two antigens. In the case of the growths studied here, it is possible that their proportions are different in each. Anti-sera obtained following immunization with C1300 contain an antibody reacting with C3H cells more frequently than is the case when 15091a is the antigen. This suggests that the former tumor contains more of antigen I, whilst absorption

TABLE VI: THE EFFECT OF STORAGE ON SERA

Days of storage	Treatment	Titred in	Dilution of serum: 2	4	8	16	32	64	128	256	512	1024
0	Nil	Saline	−	−	−	−	++	++	−	−	−	−
		50% serum	−	−	−	−	++	+++	+	+	−	−
1	Nil	Saline	−	−	−	−	−	−	−	−	−	−
		50% serum	−	−	−	−	−	−	−	−	−	−
	58.5°/C. for	Saline	−	−	−	tr	tr	tr	tr	−	−	−
	20 minutes	50% serum	−	−	tr	+	++	++	+	tr	−	−
0	Nil	Saline	c	a.c.	+++	++	++	++	++	+	+	tr
		50% serum	c	c	+++	++	++	++	++	++	++	1
28	Nil	Saline	−	−	−	−	−	±	±	±	±	tr
		75% serum	−	−	−	−	−	±	±	±	±	tr
0	Nil	Saline	a.c.	c	+++	+++	+++	+++	+++	+±	±	−
4	Nil	Saline	tr	tr	tr	+++	+++	+++		Not tested		
18	Nil	Saline	−	−	−	+	++	+++	a.c.	+++	Not tested	

experiments suggest that 15091a contains more of antigen II. This quantitative difference may explain in part the difference in the antibody response shown by C57 blacks. In man, blocking antibodies are formed more frequently against antigen Rho than against the other Rh antigens.

If one attempts to generalize on the response of mice to hyper-immunization, it is possible to say only that even if the genetic constitution of the recipient is standardized as far as possible, each antigen appears to have its own peculiarities, which again may be varied according to the manner in which it is administered. If we use the same antigen, as was done with 15091a, it is apparent that the genetic constitution of the animal producing the antibody has considerable influence on the type of serum that will be obtained.

In previous publications it was pointed out that a tumor may grow in the presence of antigenic differences between transplant and host. The phenomenon of concomitant immunity made it seem likely that antibodies might be formed under these conditions (9). In the experiments reported here these were demonstrated directly in animals that were certainly going to die from the tumors. This might seem to indicate that antibodies are of minor importance. Such a conclusion is unjustified. Death from bacterial infection may occur in the presence of high titers of antibodies. Furthermore, these animals live much longer than the naturally susceptible mice which do not form iso-antibodies; over 3 months as compared with 3 to 4 weeks. It may well be that other factors than antibodies are of importance in determining the fate of transplanted growths. If the present writer has previously over-emphasised their role (10), it was in order to point out that transplantation immunity is not some novel and mysterious process, but is fundamentally the same as immunity to infection.

It has already been shown that the agglutinins can be absorbed from a serum, leaving the protective action virtually unaltered. It would appear from this that such antibodies are of much the same significance as anti-flagellar bodies in salmonella infections, etc. However, the behavior of the sera on storage suggests another explanation, Even when stored in a frozen state there is a tendency for the agglutinins to be transformed into partial antibodies, of which the blocking antibody appears to be the final product. The mouse stores its antibodies at about 37°C. and it is not unreasonable to suppose that this transformation will be greatly accelerated under these conditions. By analogy with human iso-immunization one might expect the blocking antibody to be of the greater functional significance. If this is shown to be so, we can draw an analogy between the antibody response and leukocytosis, the "complete" agglutinins corresponding to functionally immature myelocytes. Sera such as those obtained against 15091a being the analogue of an extreme shift to the left.

SUMMARY AND CONCLUSIONS

1. The A strain of mice carries at least 2 antigenic factors in its erythrocytes that are shared by the fixed tissues and are important in transplantation.

2. It has been shown that at least 3 types of iso-antibody may be produced by the mouse: (a) ordinary iso-agglutinins, (b) antibodies that need high concentrations of normal mouse serum to cause agglutination, (c) blocking antibodies, which up to the time of writing can only be recognized by their power to inhibit agglutination.

3. Sera of normal C57 blacks may contain natural iso-antibodies of the second type.

4. The proportions in which these antibodies exist in a given serum varies with the type of tissue inoculated, the interval between inoculations, the interval between inoculation and bleeding and the genetic constitution of the host. Details of the response to inoculation with whole blood and 2 tumors (C1300 and 15091a) will be found in the text.

5. In hyperimmunized animals it is common to find a distinct pro-zone with complete inhibition of agglutination up to a high dilution.

6. Animals dying as a result of tumor inoculation may have very high titers of iso-antibodies.

7. Antibodies needing serum for agglutination are found following a relatively weak stimulus.

8. Such antibodies and blocking antibodies persist longer in the circulation than do ordinary agglutinins.

9. The factor in normal plasma or serum that enhances agglutination is thermostable.

10. On storage in the frozen state there is a tendency for antibodies to be transformed towards the blocking type of antibody.

11. Ordinary agglutinin is without protective function. It is suggested that it may mature *in vivo* to a functional antibody. An analogy is drawn between leukocytosis and iso-antibody formation. Sera with high titers of ordinary agglutinins correspond to an extreme left shift.

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The Uptake of Radiophosphorus in the Phospholipid Fraction of Mouse Epidermis in Methylcholanthrene Carcinogenesis*

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The integration of the chemical, physical, and histological changes in mouse epidermis during carcinogenesis induced by methylcholanthrene has been recently reviewed in a second summarizing report by Cowdry (4). Investigations on the lipids (13, 14), minerals (1), B vitamins (11), and succinic dehydrogenase and cytochrome oxidase (2) have been reported. Experiments are being planned which will enable us to study the uptake of radiophosphorus in the nucleoprotein, acid soluble and phospholipid fractions of mouse epidermis undergoing carcinogenesis by methylcholanthrene. In this paper we wish to describe our investigations on the uptake of P^{32} in the epidermal phospholipid fraction.

EXPERIMENTAL PROCEDURE

The procedures for shaving the mice, applying the carcinogen, and removing the epidermis from dermis have been described (2). In this study methylcholanthrene in benzene, and benzene alone were applied on alternate days for 3, 6, and 12 treatments, and the mice were sacrificed 5 days after the last application of the carcinogen or solvent. For the final stage in our series, the transplantable squamous cell carcinoma of Cooper, Firminger, and Reller was employed (3).

The phospholipid fractions of normal, benzene-treated, and methylcholanthrene-treated epidermis and of the carcinoma were extracted twice with a reflux condenser using about 25 cc. portions of a mixture of 3 volumes of alcohol and 1 volume of reagent grade chloroform. The latter solutions containing the phospholipid were evaporated to dryness on a steam bath, and the lipid was re-extracted with petroleum ether (b.p. 30 to 60°C.) The ether was evaporated on a steam bath, the residues digested in a Pyrex digestion tube with a mixture of 2 cc. of reagent grade nitric acid and 2 cc. of reagent grade perchloric acid. The digests were made up to a volume of 50 cc., and the orthophosphate content was determined on an aliquot by the method of Truog and Meyer (12) in a Coleman spectrophotometer.

To determine the rapidity of P^{32} uptake in the epidermis, solutions of P^{32} as sodium phosphate were injected subcutaneously into mice. The phospholipid fraction from mice so treated with P^{32} was extracted and digested in the same manner as described above. The perchloric acid solution containing the phosphate was diluted with a few cubic centimeters of water, neutralized with sodium carbonate, and quantitatively transferred to a 25 cc. volumetric flask. One cubic centimeter of the neutralized solution was delivered onto a small watch crystal, and evaporated to dryness in a vacuum dessicator. The resultant samples were mounted in a standard position under a "bell-jar" type of Geiger Mueller counter equipped with a thin aluminum window. Corrections for self-absorbtions were not necessitated because the sample thickness in no case exceeded a few mgm./cm. Decay corrections were made when required by response to a control sample of radioactive phosphate prepared from the same solution used for injection. The total weight of sample was determined in each case by drying the alcohol ether extracted tissue at 105°C. to constant weight. The P^{32} content was then corrected to unit dry weight of lipid free tissue so that all samples were directly comparable as to specific P^{32} content.

RESULTS

The lipid-phosphorus content of normal-untreated, benzene-treated, and of methylcholanthrene-treated epidermis and of the carcinoma is expressed as milligrams of lipid-phosphorus per 100 mgm. of dry fat-free tissue (Table I). This value for untreated epidermis was 0.163 which agreed rather well with that of the epidermis of mice which had received 3 applications of benzene (0.173), and 6 treatments with benzene (0.153). On the other hand, the phosphorus fat-free dry weight value of the epidermis which had been

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painted 3 times with the carcinogen was 0.123, a decrease of about 25 per cent from the normal. Those animals receiving 6 and 12 applications of the carcinogen had quantities of 0.120 and 0.112, respectively, which were 26 and 31 per cent less than that of the normal. In the carcinoma, the value increased to 0.311 which was nearly twice that of the normal-untreated epidermis.

TABLE I: LIPID PHOSPHORUS/FAT-FREE DRY WEIGHT RATIO OF MOUSE EPIDERMIS

Number of mice	Number of paintings	Time after first treatment to killing of mice, (*days*)	Phosphorus/fat-free dry weight ratio
		NORMAL, UNTREATED MICE	
9			0.174
11			0.161
9			0.155
29 (Total)			Average 0.163
		BENZENE-TREATED MICE	
9	3	10	0.184
10	3	10	0.167
10	3	10	0.169
29 (Total)			Average 0.173
11	6	17	0.160
10	6	17	0.168
11	6	17	0.132
32 (Total)			Average 0.153
		METHYLCHOLANTHRENE-TREATED MICE	
8	3	10	0.118
7	3	10	0.120
7	3	10	0.132
22 (Total)			Average 0.123
9	6	17	0.120
9	6	17	0.112
8	6	17	0.128
26 (Total)			Average 0.120
9	12	31	0.115
8	12	31	0.129
9	12	31	0.093
26 (Total)			Average 0.112
		TUMORS	
Tumors from sufficient animals were pooled to give 60-150 mgm. fat-free dry weight samples.			0.321 0.299 0.319 0.303 0.280 0.307 0.340 0.306 0.316 0.322
			Average 0.311

To determine the time of maximum uptake of P^{32} in normal-untreated, benzene-treated, and methylcholanthrene-treated epidermis, and of the transplantable squamous cell carcinoma, radiophosphorus as disodium acid phosphate solution was injected subcutaneously (1.0 cc. per mouse), and the mice were sacrificed from 6 to 60 hours after injection of the P^{32}. The results of this experiment, corrected to standard initial activity, are shown in Table II. The time of maximum uptake of P^{32} in benzene-treated, methylcholanthrene-treated epidermis and of the carcinoma was 12 hours, while that of the normal epidermis was at 24 hours. The uptake in the carcinoma was very high, and was maintained higher than the other tissues for 48 hours.

TABLE II: UPTAKE OF P_{32} IN THE PHOSPHOLIPID OF MOUSE EPIDERMIS

Material	Time after injection of P_{32}, (*hours*)	Counts per minute per 100 mgm. dry fat-free tissue
Normal, untreated epidermis	6	12,100
	12	14,210
	18	15,750
	24	22,640
	36	11,620
Methylcholanthrene-treated epidermis, 3 paintings	12	13,560
	24	13,640
	36	12,520
	48	10,200
Benzene-treated epidermis, 3 paintings	6	11,210
	12	18,000
	18	13,500
	24	11,210
Carcinoma	12	33,620
	24	33,810
	36	27,380
	48	24,330
	60	18,030

In Fig. 1 the specific activity (counts per minute per mgm. lipid-phosphorus) is plotted against the time. The uptake of P^{32} in the benzene-treated epidermis was rapid and maximal at 12 hours and fell quickly, while that of the normal rose slowly reaching its peak at 24 hours, but dropped at about the same rate. On the other hand, the uptake in hyperplastic epidermis and in the carcinoma was rapid and was maintained at near maximal levels for nearly 36 hours. Moreover, the rate of fall of the P^{32} was almost the same for these tissues. The specific activity of the normal epidermis at the time of maximum uptake was somewhat higher than the other tissues, but not significantly so, in view of other data to be shown later in this report.

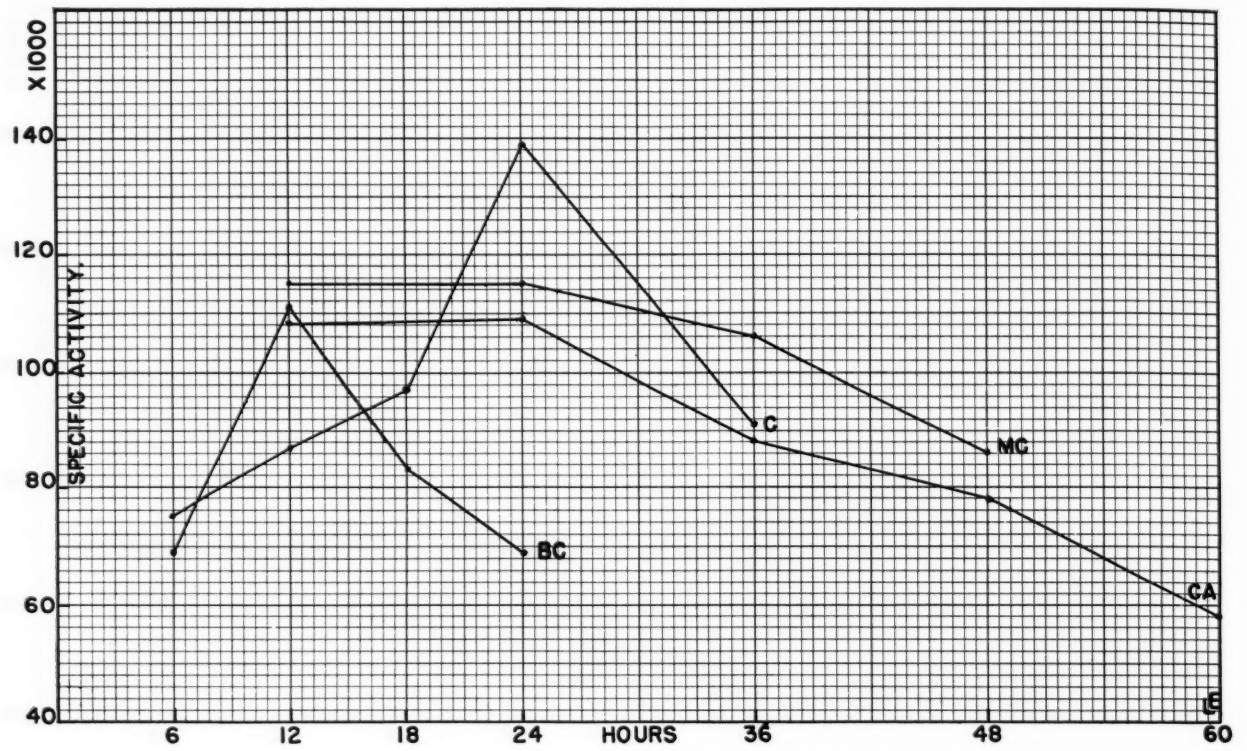

FIG. 1.—Specific activity time curves for benzene-treated (BC), normal-untreated (C), and methylcholanthrene-treated (MC) epidermis, and for the carcinoma (CA).

For the study of the influence of repeated applications of methylcholanthrene in benzene or of benzene alone on the uptake of P^{32} in the phospholipid fraction of the epidermis and of the carcinoma, the time of maximum uptake was that shown in Fig. 1.

In conjunction with the study of the time of maximum uptake of P^{32}, a series of untreated controls and of benzene-treated controls was run at the same time with the samples of P^{32} of nearly the same activity. The results (Table III) expressed as counts per minute per 100 mgm. dry fat-free tissue showed 22,118 counts for normal epidermis, whereas those for mice receiving 3, 3, 6, and 12 applications of benzene were, respectively, 22,075, 24,372, 20,641, and 20,758. Therefore, benzene alone has no appreciable effect upon the uptake of P^{32}.

In another larger experiment with another sample of P^{32}, the uptake of normal, benzene-treated, and methylcholanthrene-treated epidermis was determined (Table IV). The data are expressed as in the preceeding table. The count for untreated controls was 9,500, and for mice treated 3 times with benzene, the value was 9,320. The values for the epidermis which had been painted 3, 6, and 12 times with the carcinogen were, respectively, 8,073, 7,600, and 7,706. In the carcinoma, the uptake rose to 20,450 counts per minute. These

TABLE III: UPTAKE OF P^{32} IN THE PHOSPHOLIPID FRACTION OF MOUSE EPIDERMIS

Number of mice	Number of paintings	Time after first treatment to killing of mice, (*days*)	Counts per minute per 100 mgms. fat-free epidermis	Corrected to standard initial activity
		NORMAL, UNTREATED MICE		
5			1,064	
5			1,081	
5			926	
15 (Total)			Average 1,024	22,118
		BENZENE-TREATED MICE		
5	3	10	959	
5	3	10	1,009	
5	3	10	1,097	
15 (Total)			Average 1,022	22,075
5	3	10	1,976	
5	3	10	2,261	
5	3	10	1,856	
15 (Total)			Average 2,031	24,372
5	6	17	875	
5	6	17	785	
5	6	17	929	
15 (Total)			Average 863	20,641
5	12	31	988	
5	12	31	869	
5	12	31	1,025	
15 (Total)			Average 961	20,758

counts are considerably lower than those given in Table III, which is due to the fact that a solution of radiophosphorus of different specific activity was administered. No attempt was made to inject the same amount of P^{32} per gram body weight since the activity of our solutions varied too much for this purpose. We chose to compare the specific activities in the different experiments. The specific activity of the epidermal lipid phosphorus of the mice shown in Table IV did not differ significantly (Table V). The specific activity of normal, and benzene-treated epidermis was respectively 58,282, and 60,915, while the respective values for the hyperplastic epidermis and carcinoma were 65,635, and 65,755. The specific activity values from Fig. 1 at maximal uptake time in the normal and treated epidermis and in the carcinoma are not significantly different, and calculated ratios for the normal and benzene-treated epidermis of mice shown in Table III would be quite constant. Although the uptake varies somewhat in the different experiments, the specific activities are quite constant, which demonstrates no appreciable significance in the phospholipid turnover of the differently treated epidermises, and of the carcinoma studied here.

TABLE IV: UPTAKE OF P^{32} IN THE PHOSPHOLIPID FRACTION OF MOUSE EPIDERMIS

Number of mice	Number of paintings	Time after first treatment to killing of mice, (*days*)	Counts per minute per 100 mgms. fat-free epidermis	Corrected to standard initial activity
		NORMAL, UNTREATED MICE		
8			2,909	
8			3,038	
9			2,931	9,500
25 (Total)			Average 2,959	
		BENZENE-TREATED MICE		
5	3	10	2,493	
5	3	10	3,074	
5	3	10	2,153	9,320
15 (Total)			Average 2,573	
		METHYCHOLANTHRENE-TREATED MICE		
5	3	10	7,816	
6	3	10	8,143	
6	3	10	8,261	8,073
17 (Total)			Average 8,073	
5	6	17	2,300	
5	6	17	2,292	
5	6	17	2,848	8,000
4	6	17	2,475	
5	6	17	3,225	
4	6	17	2,575	7,200
28 (Total)			Average 2,619	7,600
5	12	31	6,811	
5	12	31	7,157	
5	12	31	8,071	
5	12	31	8,785	7,706
20 (Total)			Average 7,706	
		TUMORS		
			15,305	20,500
			15,217	20,000
Tumors from sufficient animals were pooled to give 60-150 mgm. fat-free weight per sample			15,138	19,900
			14,303	18,700
			17,333	22,600
			16,033	21,000
			Average 15,555	20,450

TABLE V: RATIO OF COUNTS PER MINUTE PER 100 MGM. DRY FAT-FREE TISSUE AND MGM. LIPID PHOSPHORUS ON THE SAME BASIS OF REFERENCE (SPECIFIC ACTIVITY)

Material	a Counts per minute per 100 mgm. dry weight	b Mgm. lipid phosphorus per 100 mgm. dry weight	$\frac{a}{b}$ (specific activity)
Normal, untreated epidermis	9,500	0.163	58,282
Benezene-treated epidermis, 6 paintings	9,320	0.153	60,915
Methylcholanthrene-treated, 3, 6, and 12 paintings	7,745	0.118	65,635
Carcinoma	20,450	0.311	65,755

DISCUSSION

In an investigation of the rate of phospholipid turnover in 4 types of transplantable tumors in mice (a mammary carcinoma, a lymphoma, a lymphoblastoma, and sarcoma 180), Jones, Chaikoff, and Lawrence (6, 7) observed that the phospholipid turnover resembled that in tissues such as liver, kidney, and intestine, rather than in tissues like muscle or brain, which are less capable of regeneration and growth. They also observed that the rate of turnover for the types of tumors was not uniform, but that each displayed a characteristic activity. In another study, the same authors demonstrated that the total phosphorus turnover of three neoplastic tissues (a mammary carcinoma, a lymphoma, and a lymphosarcoma) showed a high and rapid uptake of P^{32} in the early intervals after its administration, and that the malignant tumors had a pronounced capacity for retaining P^{32} for a long time in contrast to normal tissues with equal levels of P^{32} (8). In our study on epidermal carcinogenesis, the specific activity time curves showed no appreciable difference at maximum uptake time between normal benzene-treated, and methylcholanthrene-treated epidermis and the carcinoma. However, the specific activity time curves of the carcinoma and hyperplastic epidermis, the latter being chemically precancerous (2), was quite similar in that both re-

tained their radiophosphorus at a higher level for a longer period of time, and decreased at nearly the same rate.

From experiments on the rate of turnover by lecithins and cephalins of rat carcinosarcoma 256, Haven (5) concluded that the rapid turnover of the lecithins indicated participation of lecithins in metabolic activities. The slower rate of turnover by cephalins suggested relationship to cell structure. Marshak (9) has made a detailed investigation of the uptake of P^{32} by the nuclei of liver and tumor cells, and has shown, among other things, that the nuclei of tumor cells accumulate more P^{32} than do normal liver nuclei. This may be due to greater mitotic activity of the tumor cells and not to the possession by them of a different and characteristic type of metabolism (9). Investigations on tumors employing labelled phosphorus have dealt chiefly with the leukemias (references cited by Scott [10]) which are obviously quite different from carcinomas in our experiments.

SUMMARY

The effect of methylcholanthrene on lipid phosphorus and on the uptake of phosphorus in the lipid fraction of mouse epidermis undergoing carcinogenesis induced by methylcholanthrene was investigated by the use of P^{32}. The carcinogen caused a drop of 30 per cent in the lipid phosphorus content of the epidermis, but in the carcinoma, the lipid phosphorus content was nearly twice that of the normal. The specific activity time curves of normal, benzene-treated, and methylcholanthrene-treated epidermis and of the carcinoma showed no appreciable difference in the specific activities at the time of maximum P^{32} uptake. The rate of uptake in the hyperplastic epidermis and in the carcinoma was rapid, these tissues retained their labelled phosphorus for a considerable time, and both showed a similar fall in activity. The uptake in benzene-treated epidermis was at a maximum at 12 hours, while that of the normal was slower and at a maximum at 24 hours. The rate of fall in each was quite rapid and similar. Other experiments demonstrated no significant difference in the specific activities of normal epidermis or of epidermis treated 3, 6, and 12 times with benzene alone or with the carcinogen in benzene on alternate days during 10, 20, and 30 days. Although the carcinoma had a high and rapid uptake of P^{32}, its specific activity was similar to that of the treated epidermis.

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The Carcinogenic Activity of 2-Acetaminofluorene Characteristics of the Lesions in Albino Rats*

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Attention has been called to the development of a variety of tumors in rats and mice after the administration of 2-acetaminofluorene by mouth (2, 11). Species differences in susceptibility and in distribution of proliferative lesions have been noted (1, 12), but animals of a single strain have shown fairly consistent development of characteristic lesions. We have used principally albino rats originally of the Stanford strain (11), so the discussion to follow will deal with tumors in these highly susceptible animals.

It is probable that the tumors are induced not by the acetaminofluorene itself, but by some derived product, possibly aminofluorene (13). The nature of the resultant tumors is similar whether acetaminofluorene or aminofluorene is administered to rats.

Focal proliferation of a number of different types of epithelium has appeared following continued feeding of acetaminofluorene in concentrations in the diet ranging from 0.5 per cent to 0.004 per cent. The other characteristics of the diet have been described previously (11). It is not necessary to continue the administration of the substance until the time of appearance of lesions; a period of administration as short as 25 days may be followed months later by the development of tumors (12).

Since the first report further observations have resulted in the detection of a few additional types of lesions and have given more information concerning the frequency of proliferative changes. Tissues from 108 rats which had lesions apparently due to acetaminofluorene have been studied histologically. The animals died or were killed at various intervals after administration of the substance in different quantities, so that the circumstances under which the tumors developed are not closely comparable. Since some of the animals, including all that were fed more than 0.125 per cent acetaminofluorene in the diet, were killed or died early in the course of an experiment before advanced lesions appeared, it was decided to include in this report only those rats which, because of the appearance of multiple lesions, had presumably received a thoroughly effective exposure to the compound. There were 84 such animals, 22 males and 62 females, and in most of them opportunity for the development of multiple lesions was great because they lived until their condition was poor or until large external lesions had appeared. The period of observation ranged from 104 to 695 days following the institution of the augmented diet, which contained from 0.008 per cent to 0.125 per cent acetaminofluorene for intervals not always as long as the observation periods.

TABLE I: INCIDENCE OF THE MOST COMMON LOCAL HYPERPLASTIC AND NEOPLASTIC LESIONS IN 84 RATS (22 MALE AND 62 FEMALE) IN WHICH TWO OR MORE TISSUES WERE INVOLVED

Organ	Number of animals with lesions		Total incidence, %	Proportion malignant, %
	Male	Female		
Liver	21	57	93	22
Bladder	16	51	80	21
Lung	8	24	38	16
Head	5	21	31	92
Breast	3#	23#	31	31
Kidney	3	17*	24	25
Uterus		13†	21	8
Thyroid	2	9	13	0
Gastrointestinal tract	1	4	6	60

Includes one subcutaneous fibroma.
*Includes one carcinoma of the upper ureter.
†Includes one sarcoma.

Table I indicates the incidence and distribution of hyperplastic changes in the 84 rats that had more than one type of lesion. Since the acetaminofluorene was administered in different dosages in different animals, the indicated incidence of the various lesions is of value principally in showing the relative frequency of the different changes. After autopsy and fixation of the tissues in 4 per cent formaldehyde, histological sections stained with hematoxylin and eosin were prepared from all grossly abnormal tissues except for 2 mammary

*The experimental work on which this paper is based was completed in 1942, but because of the pressure of other work the authors were unable to prepare their results for publication.

tumors, which were lost. Many organs without gross lesions were also sectioned. In all there were sections from the liver of 83 animals, the urinary bladder of 79, the lung of 78, the kidney of 77, the pancreas from 76, the thyroid gland from 60, the ovary from 37, and the uterus from 32 animals. Numerous sections of heart, spleen, testis, stomach, intestine, thymus, hypophysis, salivary gland, skin and bone were examined histologically but they showed no proliferative lesions in addition to those identified by gross examination. Thirty-nine brains examined histologically have shown no lesions suggesting that described as a glioma by Lopez (7), and no other abnormalities have been found in them.

There was much variety in the degree of change in the affected organs of different animals, ranging from focal proliferative lesions which were visible only microscopically, to large grossly recognizable nodules which greatly distorted the shape of the involved organs. Multiple nodules in the liver commonly increased its size by more than 100 per cent. The lesions have not been classified according to size and no distinction between nodular hyperplasia and adenoma formation has been made because no basis has been found for anatomical separation of these states among the experimental lesions. However, an attempt has been made to classify lesions as malignant if there was infiltration of abnormal cells among pre-existing tissue elements. In cases where there was only questionable slight hyperplasia the tissue has been listed as unaffected, and no lesions are recorded as malignant unless distinct infiltration of adjacent tissues could be recognized histologically. It is likely that some instances of malignancy were not detected by this method. In general, the extent of the hyperplasia was great and cell differentiation was poor when infiltration of tumor cells was present, but there were several exceptions. Most of the tumors were composed of epithelial cells, although 5 animals had leukemia and 2 had sarcomas. One of the latter arose in the muscles of the leg and the other developed in the uterus.

The following paragraphs present the types of tissue change that have occurred with sufficient frequency to suggest that they are specific effects of the administration of acetaminofluorene.

Liver nodules.—These were the commonest and most prominent lesions, causing gross deformity of the liver in more than half of the animals. The lesions were always multiple and some reached a diameter of 3 or 4 cm. Most of the nodules were composed of cords of hepatic cells which were similar to, but distinctly different from the normal liver cells (Fig. 1). The cords were sometimes irregular and did not form a lobular pattern, although they bordered prominent sinusoidal spaces. No portal connective tissue spaces could be seen within the nodules. The cytoplasm of the abnormal cells had a varied appearance. In some parts it was more dense and more uniformly stained than normal, but in others it had a reticulated appearance or was distinctly vacuolated. Its volume per cell was distinctly greater than normal. The nuclei were large and quite varied in size and intensity of staining. Nucleoli were large and sometimes multiple. Mitotic figures were prominent in some of the larger nodules. Bordering the sinusoids there were occasional prominent endothelial cells resembling Kupffer cells. In a few places these contained a little light brown pigment that did not give the Prussian blue reaction for iron. There was no evidence that the abnormal nodules of hepatic cells were drained by bile ducts, yet most of them were not distinctly jaundiced. Several of the larger nodules, however, were grossly yellowish and they showed scattered small deposits of iron-free yellow pigment not only in the endothelial cells, but also in some of the hepatic cells.

In many of the livers there were additional nodules of quite different appearance. These were composed of small duct-like structures and cysts which had a lining of simple low columnar or flattened epithelium (Fig. 2). Separating these structures was a little dense fibrous tissue. Certain of the cysts were multilocular and among them in a few places were groups of hepatic cells which appeared to have been isolated from pre-existing

DESCRIPTION OF FIGURES 1 TO 6

FIG. 1.—Small nodule of hyperplastic hepatic cells adjoining normal liver tissue. At the edge of the nodule on the right is a central vein. Mag. × 120.

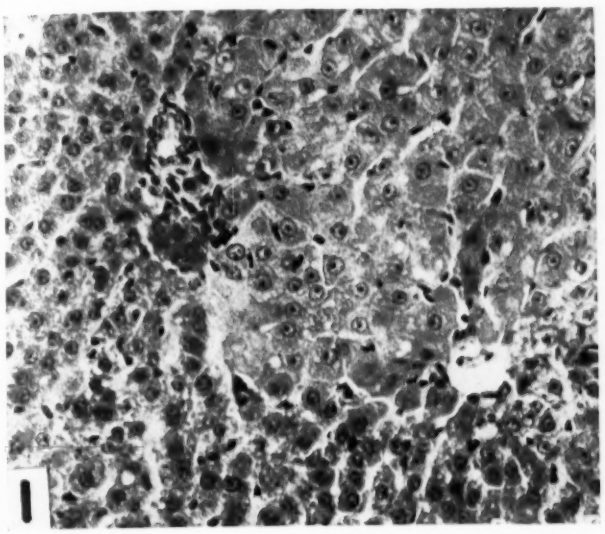
FIG. 2.—Nodule in liver composed entirely of cysts lined by low columnar epithelium. Mag. × 48.

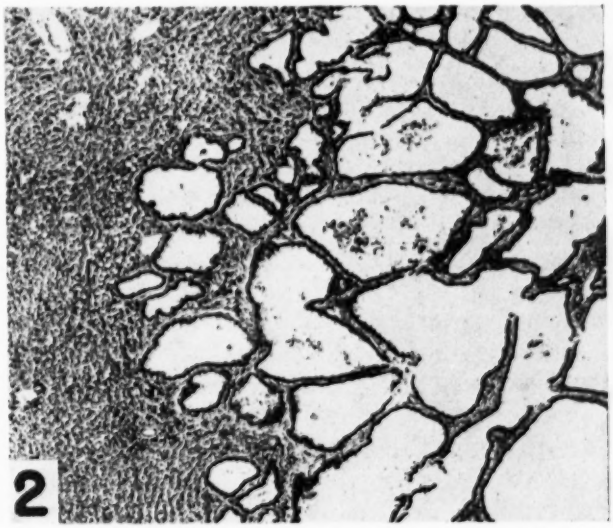
FIG. 3.—Lung nodule formed by proliferated epithelial cells within alveoli. This is apparently benign. Mag. × 48.

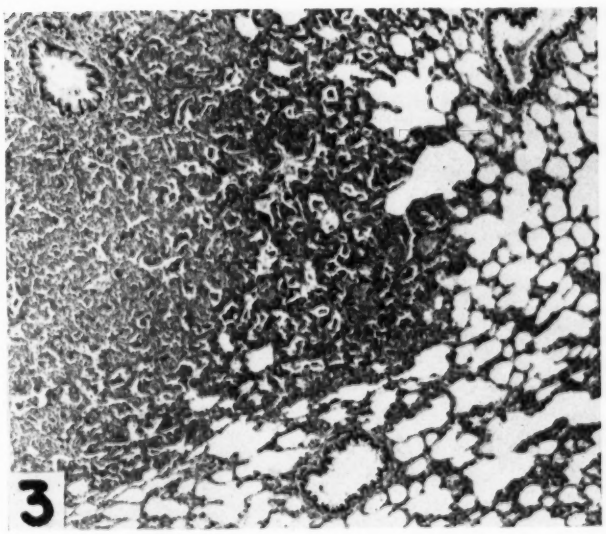
FIG. 4.—Similar lung tumor. Mag. × 120. Clumps of cells in alveolar lumen can be distinguished from layer of alveolar lining cells.

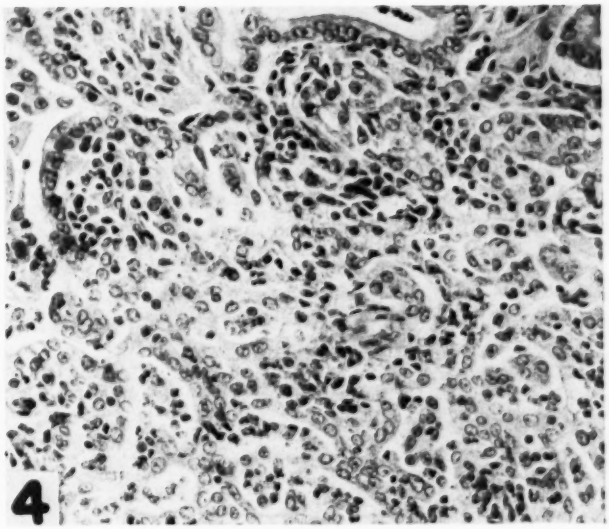
FIG. 5.—Epithelial tumor arising from renal pelvis. Narrow rim of atrophic kidney tissue borders it on the left. Mag. × 48.

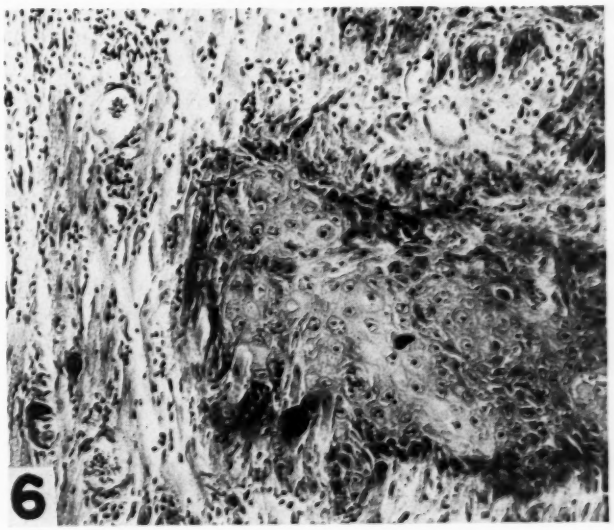
FIG. 6.—Border of a malignant tumor of squamous epithelium arising adjacent to the deep end of the external auditory canal. Mag. × 120. Projections of tumor cells have infiltrated skeletal muscle.











FIGS. 1-6

liver tissue. The cells forming these cysts were more uniform than those of many of the solid nodules.

Frequently the same liver contained nodules of different types, and when one nodule showed malignant changes, others appeared benign. In the presence of large tumors, other parts of the liver regularly contained microscopic groups of enlarged cells forming discrete but non-encapsulated nodules. In some of the nodules of hepatic cells there were numerous small spaces often reaching a diameter of 1 mm. These were bordered by flattened cells and produced an appearance suggesting abnormal dilated bile ducts. These duct-like structures appeared to be integral parts of the hyperplastic nodules, although the manner of their development was not determined. These nodules containing hepatic cords and glandular structures correspond to the adenohepatomas described by Opie (8) after feeding *p*-dimethylaminoazobenzene. Two of the malignant liver tumors contained duct-like structures associated with other cells resembling hepatic cells.

Several of the large partially necrotic lesions were accompanied by infiltration of a variety of inflammatory cells but this was not observed in small lesions which were likewise unassociated with any fibrosis of the liver substance.

Nodular proliferation of epithelium in the lungs.—In lungs from 22 rats there was a characteristic sort of focal epithelial proliferation similar to that which has been observed in mice by many investigators. In 17 animals multiple lesions were found. A number were demonstrable only histologically, and since from most animals only one or two sections of lung tissue were prepared, it is certain that other undisclosed small lesions were present. Inflammation was prominent in some lungs and it is possible that instances of nodular epithelial proliferation were concealed by it. However, many of the nodules were not associated with inflammation. The lungs from control animals have not been examined by serial sectioning, but in numerous sections from old animals which did not receive acetaminofluorene, no lesions of this type have been seen.

Most of the pulmonary nodules were less than 4 mm. in diameter. They were localized though not encapsulated, and their appearance suggested a benign proliferative process. The principal constituents were abnormal epithelial cells within the alveolar spaces (Figs. 3 and 4). The pulmonary structure persisted within the nodules but the alveolar spaces were smaller than those elsewhere. Each alveolus in the altered region was lined by a layer of simple cuboidal or low columnar cells and in most of the spaces there were also small solid masses of polygonal, similarly stained cells. A small cleft usually separated these from the layer bordering the alveolar wall. The proliferated cells had nearly round nuclei with prominent small chromatin particles but no large nucleoli. Scattered nuclei were hyperchromatic and there were a few mitotic figures. The cells commonly appeared columnar on one side of an alveolus and cuboidal or even flattened on another. In a few places, clumps of 4 or 5 nuclei were very closely placed, suggesting formation of small syncytial masses. Most of the cells had scanty, slightly acidophilic cytoplasm. The appearance suggests that these may have been derivatives of normal alveolar lining cells. Small bronchi were present within some of the nodules but their epithelium was distinctly different from that of the intra-alveolar cells. The lesions in our rats were similar to those induced in mice with dibenzanthracene and methylcholanthrene by Grady and Stewart (5), who have presented satisfactory evidence based upon the study of serial sections, that the proliferating cells of their tumors originated in the alveoli.

The lungs from 5 additional acetaminofluorene-treated rats contained larger masses composed of less uniform cells which had destroyed lung tissue and had penetrated through the pleura or into large bronchi. The cells of these malignant tumors were small, they were arranged in small irregular clumps and strands, and their appearance suggested an epithelial origin. In places they showed a resemblance to the cells of the benign lesions. A lung from one additional animal showed a small papillary intrabronchial benign tumor in which the epithelium was similar to that of the bronchial wall.

It was suggested in our original report that the metaplastic squamous epithelium lining bronchi in certain regions of chronic inflammation in some lungs might be a specific effect of acetaminofluorene feeding. Similar regions of metaplasia have been found in additional animals, but they have also appeared in a number of old rats with chronic pulmonary inflammation which did not receive any carcinogenic substance and which showed no other lesions like those in the acetaminofluorene-treated animals. Therefore, this bronchial epithelial metaplasia cannot be regarded as a specific effect.

Epithelial proliferation in the urinary tract.—With the exception of a benign tubular adenoma in one kidney, all of these lesions have been derived from transitional epithelium of the bladder, renal pelvis and ureter. The lesions were always focal

and were usually multiple. There was wide variation in their size as well as their character. Small lesions sometimes consisted of no more than slight local thickening. The cells in the thickened zones were usually enlarged and arranged less regularly than in normal transitional epithelium. In many instances they resembled stratified squamous epithelium and some showed various degrees of keratinization. Most of the nodules were flat but elevated papillary lesions were common. Malignancy was recognized by deep penetration of irregular cords of tumor cells, which were usually less differentiated than the cells of the benign lesions. They frequently resembled stratified squamous epithelium rather than transitional epithelium. Some surrounded small foci of keratinization.

The hyperplastic changes were much more frequent and more prominent in the bladder than in the remainder of the urinary tract. Several instances previously reported as hyperplasia of the kidney pelvis are omitted from the present classification since they were minimal. Three carcinomas of the renal pelvis were present, although infiltration of adjacent tissue was not extensive. All these tumors were papillary in some parts; 1 was composed of keratinizing squamous epithelium, whereas in 2 the tumor cells resembled transitional epithelium (Fig. 5). A single tumor in the region of the upper end of one ureter was a squamous cell carcinoma. Since routine histological examination of the ureters was not made, no knowledge of the incidence of small ureteral lesions is available.

Subcutaneous tumors of the side of the head.—These tumors all appeared just anterior to the ear and were covered by intact movable skin when they were small. They were composed largely of squamous cells in abnormal arrangement. Usually the central part contained a cavity filled with desquamated keratinized material. The wall was formed by irregular masses of stratified squamous epithelial cells of varied size and staining reaction. These were supported by thin fibrous strands and frequently surrounded small masses of keratin. At the periphery in all but two instances there was infiltration of adjacent structures by tumor cells (Fig. 6), and the appearance was characteristic of squamous cell carcinoma. The two apparently benign lesions were papillary tumors nearly 1 cm. in diameter lying within cysts which occupied the same position as the infiltrating tumors.[1] The cyst walls were lined by stratified squamous epithelium which had a smooth outer surface except for some blunt rounded projections of epithelium and a number of protruding lobules of sebaceous gland tissue in one place. The papillary portion of these tumors protruded into the cyst cavity and was composed of long thin folds covered by keratinizing stratified squamous epithelium. Parts of the cyst were packed with desquamated keratinized material.

The origin of all of the tumors of the head was apparently the same. They all developed in the same location and had a similar appearance. Although they sometimes perforated the auditory canal and were always closely attached to it, the lining of the canal was intact over several tumors except for a single small round hole in each case. The largest of these openings was 2 mm. in diameter; each had a smooth border and led into a cavity within the tumor. These openings suggested dilated duct orifices and together with the other features of these tumors, pointed to a probable origin from some adjacent accessory structure, rather than from the lining of the auditory canal. The most likely origin is the sebaceous glands which are normally prominent adjacent to the auditory canal in this region. The skin of the head was freely movable over the smaller lesions and can be eliminated as the point of origin even though the large tumors frequently produced ulceration of the skin. Parotid gland tissue was present adjacent to some of the tumors but there was a separating layer of fibrous tissue and the appearance did not suggest an origin from this tissue.

Tumors of the breast.—In our original report the mammary origin of several subcutaneous glandular tumors was not established with certainty, although it was suggested as the most likely possibility. In the animals studied subsequently, it has been possible to identify mammary tissue immediately adjacent to the tumors and there have been several instances of irregular hyperplasia of mammary tissue without distinct tumor formation (Fig. 7). The structural similarity of some of the subcutaneous tumors to portions of the hyperplastic mammary tissue provides further evidence that the tumors were of mammary origin.

In the hyperplastic glands there were usually irregular groups of mammary ducts, frequently somewhat dilated, and among some of them there was more than the usual amount of fibrous tissue, showing mild lymphocytic infiltration. Frequently there were prominent lobules of small acini. Thirty-two grossly visible tumors developed in 26 animals. Most of these were composed of irregular small glandular structures resembling mammary

[1] In several additional animals killed when tumors of this type were small, similar apparently benign lesions have been found.

ducts, lined by cuboidal or pseudostratified epithelium and scattered through different amounts of fibrous stroma. Some contained groups of structures resembling acini (Fig. 8). In some the connective tissue was prominent, as in fibroadenomas, but since there was no sharp dividing line among 19 of these benign tumors, they have all been classed as adenomas. One of the smaller lesions lay in a cyst-like space about 1 cm. in diameter lined by low columnar epithelium which was frequently piled up to a layer several cells thick. The intracystic nodule was 3 mm. broad and was attached on one side. It was composed of small irregular glands in a little cellular stroma.

Nine additional tumors were classed as malignant. These showed less uniform glandular structures than did the benign tumors, and there were commonly thin cords of atypical epithelial cells without demonstrable lumen. Two of the tumors contained cystic spaces lined by columnar cells and enclosing protruding masses of glandular tumor tissue, suggesting pre-existing papillary adenomas.

Two benign subcutaneous tumors were composed only of fibrous tissue without any glands. The origin of these is not certain, although their location was similar to that of the glandular tumors, and bordering the one which appeared in a male rat was a layer of mammary tissue. Two other subcutaneous tumors apparently of mammary origin were not examined histologically because the tissue was lost. They have been classified as benign lesions.

Nearly all the subcutaneous tumors developed in female rats, but 3 appeared in males. None of the tumors in the males has been regarded as malignant.

These observations indicate that the mammary gland in acetaminofluorene-treated rats undergoes changes comparable to those of other organs showing nodular hyperplasia in association with, and presumably preceding, tumor formation. The frequency of this hyperplasia is not known, since the mammary glands were not subjected to routine histological study.

Nodular proliferation of endometrial glands.—Eight uteri from the experimental animals showed local enlargements up to a diameter of 5 mm., and of 32 uteri which were examined histologically, 13 showed irregularities of the endometrium. These lesions all appeared in animals surviving for more than 250 days following the onset of treatment; most of the animals were more than 400 days old. It is possible that ageing played a more important part in the development of these changes than in most of the others observed in this group of animals, although comparable alterations have not been seen in other old rats.

Some of the glands in the altered zones resembled normal endometrial glands. They were small, fairly uniform, and lined by a single layer of cuboidal or low columnar epithelium. There was an accompanying increase in endometrial stroma, which sometimes contained a few scattered lymphocytes and brown pigment-filled macrophages, suggesting that bleeding had occurred. Not infrequently some glands were dilated to a diameter of more than 1 mm. These cyst-like spaces contained a small amount of eosinophilic coagulum and their lining epithelium was usually flattened. The proliferation was eccentric with respect to the lumen and the myometrium was expanded over the surface of the proliferated nodules (Fig. 9). There was no evidence of malignancy. The lower portion of one uterus was destroyed by a large sarcoma composed chiefly of spindle-shaped cells. It apparently arose in this organ.

Thyroid gland nodules.—In our first animals no proliferative nodules were noted in the thyroid gland. Bielschowsky (3) has stated that acetaminofluorene alone does not evoke thyroid hyperplasia. However, in 11 of the animals more recently studied and included in the present series there was distinct nodular irregularity of the histological structure of this gland. A possible factor of importance is that these animals were older at death than most of the animals studied previously. In four glands the nodular appearance was due principally to variation in size of follicles and in amount of colloid contained. There were several distinct but not encapsulated nodules up to 1 mm. in diameter (Fig. 10). Among abnormally large colloid-filled follicles in the nodules there were usually small empty follicles. The proliferative nature of the process is evidenced by epithelial cells of normal or even increased height forming the large follicles. In five adenoma-like nodules much of the epithelium was composed of tall

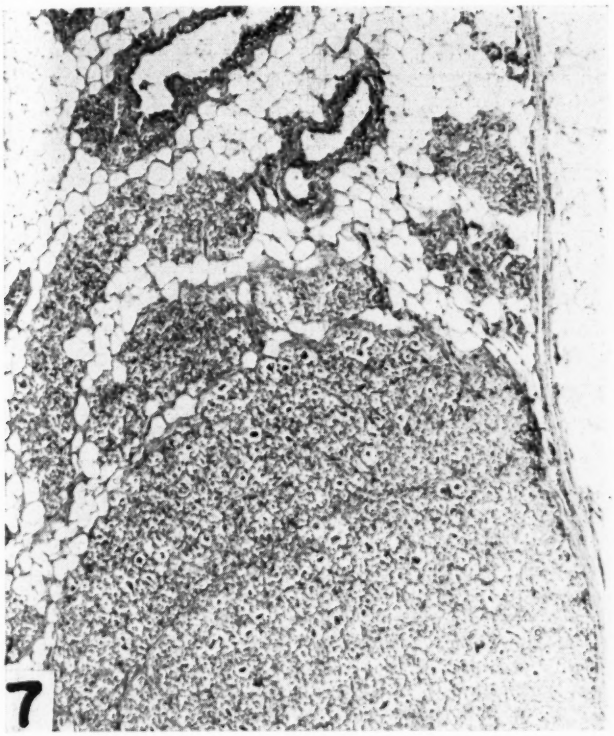
DESCRIPTION OF FIGURES 7 TO 10

FIG. 7.—Irregular hyperplasia of mammary tissue without formation of distinct tumor. Mag. × 48.

FIG. 8.—Benign fibroadenoma of mammary glands, remnant of which appears above the tumor. Mag. × 120.

FIG. 9.—Nodule of hyperplastic endometrium. This presents no evidence of malignant growth. Mag. × 48.

FIG. 10.—Nodule in thyroid gland formed by large follicles with abnormally tall lining epithelial cells. Mag. × 120.



FIGS. 7-10

columnar cells. It formed projecting folds into the lumen of moderately large collapsed follicles which contained very little colloid. The structure in these nodules had little resemblance to the normal thyroid, but their origin in this organ seems certain. Several lay deep in the substance of the thyroid gland and none were situated upon the surface as would be expected if they were derived from the parathyroid glands. These in some sections were demonstrable as separate structures. Tumors of parathyroid glands, which have been reported after acetaminofluorene administration by Heiman and Meisel (6), have not been recognized in our animals.

Tumors of the stomach and intestine.—One small adenocarcinoma of the stomach was situated beneath a shallow ulcer 2 mm. broad in the pyloric zone where a mass of atypical small glands extended into the submucosa (Fig. 11). There was also heavy infiltration of small mononuclear cells. There was not much variation of the tumor cells but the structures formed did not closely resemble normal mucosal glands. They reached the muscularis but did not penetrate it. The tumor cells contained a number of mitotic figures. Another glandular tumor of the submucosa of the stomach has been classed as an adenoma because the tubular structures forming it were more uniform than those of the tumor described above and it formed a well circumscribed, though not encapsulated, nodule.

A somewhat similar tumor was situated in the ileum of another animal. It lay principally in the submucosa and elevated the mucosa, which was not sharply distinguishable from the tumor in some places. Most of the tumor cells contained vacuoles and at the deep margin there were several glands containing Paneth cells. Although this tumor reached the muscularis, it had a sharp border and has been classified as benign.

The colons in 2 animals contained extensively infiltrating adenocarcinomas composed of moderately irregular, coarse, glandular structures with a lining of columnar epithelium. These tumor cells penetrated the entire thickness of the intestinal wall (Fig. 12). In one of these animals there were multiple metastatic growths.

Changes in the pancreas.—In our original report, microscopic nodules of prominent pancreatic acini were described in more than half of the animals in which the pancreas was examined histologically. There was also one carcinoma apparently of pancreatic origin. These lesions have been seen much less frequently in subsequent animals and no further instances of tumor of this organ have been found. There appears to be little doubt of the proliferative character of this change when the lesions are marked, but review of the sections from the original series throws doubt upon the significance of those changes reported as slight. Further observations are necessary to determine the importance of the pancreatic changes. In an accessory lacrymal gland of one rat a tiny sharply defined group of slightly enlarged acini resembled the pancreatic lesions. In several others clusters of tiny cystic spaces were seen in the pancreas, but these were not numerous and it is not possible at present to relate them to the acetaminofluorene feeding.

Leukemia.—Five of the animals had leukemia, with infiltration of multiple organs. In each case the liver and spleen were involved. Leukemic cells were present in all parts of the liver lobules, and many cells lay within the sinusoids. The appearance of these abnormal cells suggested that they were myelogenous, although studies of the cells in smears have not been made. The incidence of leukemia in the acetaminofluorene-treated animals is considerably higher than that in control animals of our colony, but leukemia has been observed frequently under other experimental conditions (13), and therefore the relation of leukemia to the acetaminofluorene feeding is less distinct than in the case of most of the other lesions described above.

Miscellaneous proliferative lesions.—A sarcoma situated among the leg muscles was described in our original report. The other sarcoma that we observed is discussed in conjunction with the uterine changes. The development of only 2 sarcomas in contrast to 76 malignant epithelial lesions emphasizes the relative rarity of tumors in the connective tissue of animals fed this agent. A malignant tumor in the mediastinum of one animal infiltrated the pericardium extensively to form a firm white layer 2 to 3 mm. thick completely en-

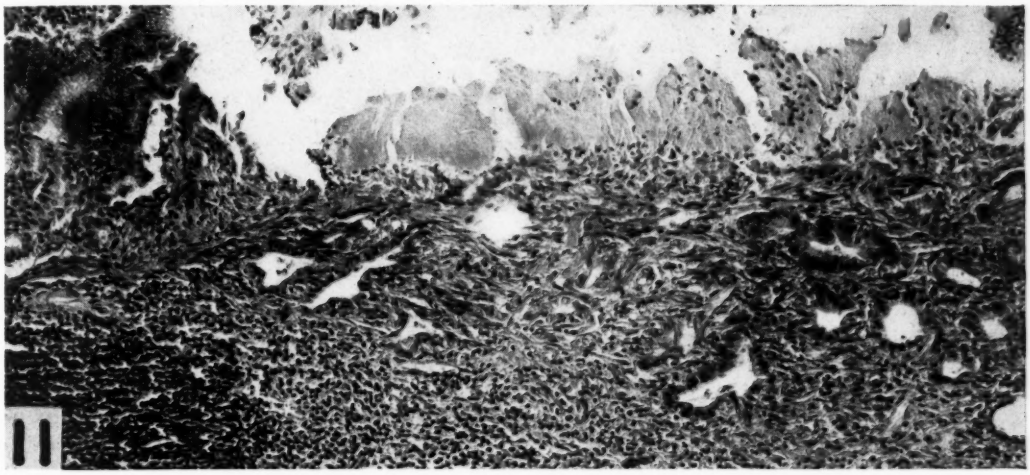
DESCRIPTION OF FIGURES 11 TO 14

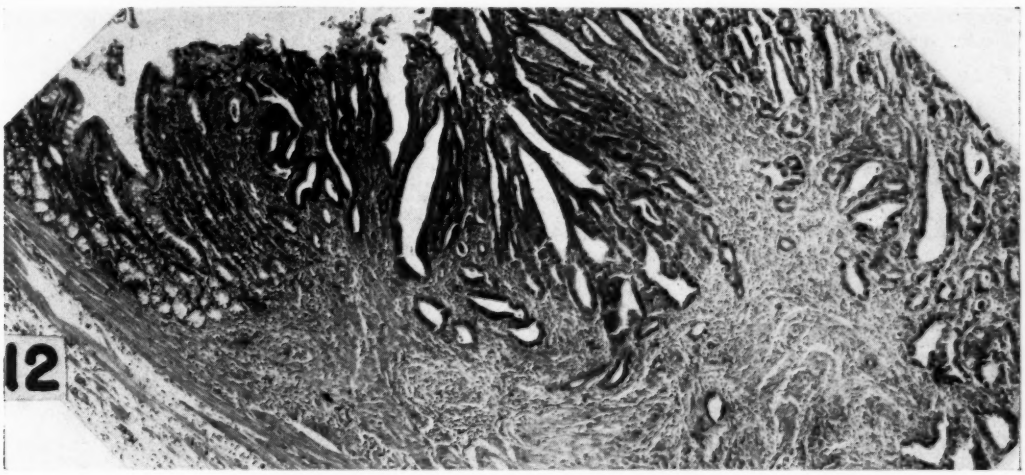
FIG. 11.—Infiltrating adenocarcinoma at base of ulcer in stomach. Mag. × 120.

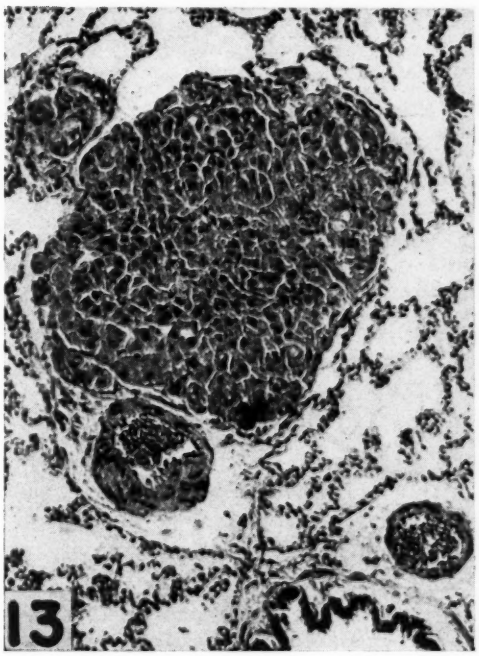
FIG. 12.—Adenocarcinoma of colon which has penetrated all layers of the intestinal wall (right). A piece of unaffected mucosa is seen at left. Mag. × 48.

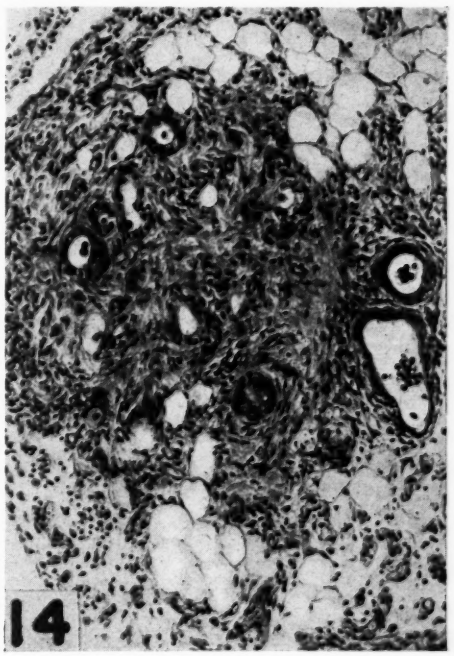
FIG. 13.—Metastatic nodule in lung from malignant liver tumor. Metastatic cells are arranged like those of the primary lesion. Mag. × 120. Two arteries and a bronchus are shown.

FIG. 14.—Metastasis in the omentum of adenocarcinoma from lesion of colon illustrated in Fig. 12. Mag. × 120.









FIGS. 11-14

casing the heart. The tumor cells were polygonal in places, but elsewhere they were elongated and spindle-shaped. Around a number of small necrotic foci the cells were larger and had a palisade arrangement. Similar palisading of the cells occurred at the surface of the epicardium. The cells were not uniform, many contained mitotic figures, and groups of them frequently lay within small vessels bordering the myocardium. It is suggested that this is a tumor of the thymus, although proof of its origin has not been obtained.

Metastasis from malignant tumors.—Metastatic lesions were found in 13 of the 52 animals bearing carcinomas. These secondary tumors were usually multiple but were of only 2 types. No animal showed metastasis from more than 1 tumor. In 11 instances metastasis occurred from liver tumors. The secondary nodules, which were structurally like the hepatic cell tumors in the liver, were found in the lungs (Fig. 13) of 8 rats, in the peritoneum and adjacent tissues of 4, and in the lymph nodes of 1 animal. No metastatic nodule contained any cyst-like structures. The other type of metastasizing tumor was adenocarcinoma (Fig. 14). Secondary growths of this type occurred in only 2 animals and were limited to tissues covered by peritoneum. In one the primary tumor arose in the colon and in the other the primary tumor was not found.

None of the tumors of the breast, urinary tract, lung or head, and neither of the 2 sarcomas, had spread to distant sites, although in several instances small satellite tumor nodules were seen adjacent to a primary tumor.

DISCUSSION

The present discussion of tumors resulting from the feeding of acetaminofluorene has been limited to their appearance in rats, but we have observed many of the same types in several strains of mice subjected to similar treatment. The frequency of involvement of particular organs differed with species and with different mouse strains, but when they appeared in mice the lesions were like those of the rat, similarly located (12).

It has been mentioned above that acetaminofluorene may not itself be the active carcinogenic principle. Bielschowsky (2) has reported the appearance of tumors when aminofluorene is applied to the skin. Histological study of tissues from 6 rats and 7 mice developing lesions after aminofluorene feeding has revealed lesions that are essentially the same as those produced by acetaminofluorene (5).

Most of the distinct tumors observed have appeared in organs which were also the site of frequent irregular hyperplasia of cells of the same type as those producing the tumors. In each such organ it has been impossible to draw a sharp line of distinction between non-neoplastic hyperplasia and tumor formation. The hyperplasia never involved all parts of an organ or tissue uniformly, but appeared only in foci, which suggests that there was gradual development of some of these foci until large, well defined nodules were formed, or until sufficient penetration of adjacent tissues had taken place to classify the process as malignant growth. In portions of lesions which showed such "invasion," the cellular structure was sometimes indistinguishable from that of other lesions which were localized. No evidence was seen to suggest a sudden transformation of hyperplastic to neoplastic cells, or of benign to malignant cells. Many authors have expressed similar conclusions with respect to experimental skin tumors and Willis (9, 10) has collected evidence suggesting that human skin tumors do not arise by a single sudden transformation of epithelial cells.

The great frequency and invariable multiplicity of nodules in the liver may be the result of greater susceptibility of the liver cells to the effect of the carcinogenic agent or to more intimate contact of the agent with the liver cells than with those of other organs. Our observations do not clarify this problem. The findings of Bielschowsky (3) who reported that combined administration of allylthiourea and acetaminofluorene will produce benign and malignant tumors of the thyroid in rats, suggest that some factor in addition to the acetaminofluorene is important in the localization of the carcinogenic effect, and that this element may be provided or enhanced by certain types of stimulation of tissue growth. While it is possible that such factors may play a part in the development of all tumors following acetaminofluorene administration, it is impossible at present to identify them. The livers in our animals did not show frequent cirrhosis such as that found after feeding *p*-dimethylaminoazobenzene to rats, and we have seen no other morphological changes that might suggest a predisposing condition. Similarly, in other organs which were the site of proliferative changes, there was no reason to suspect any specific predisposing factor. Inflammation was associated with some of the pulmonary lesions, but a number were entirely without evidence of inflammation. Also, a number of inflammatory lesions in the lungs and elsewhere were not accompanied by any unusual proliferative changes.

The rarity of the development of tumors in any but epithelial cells following the administration of

acetaminofluorene, and the repeated localization of lesions in the same organs, suggest a specificity of the carcinogenic effect, possibly related to the metabolism or excretion of the substance. On the other hand, the appearance of nodules of two types of proliferating cells in frequent association in the same liver emphasizes the lack of complete specificity of the carcinogenic agent. The possibility that the agent acts merely by acceleration of a latent tumor-forming capacity of the treated animals, as has been visualized by Engelbreth-Holm (4) and others, cannot be excluded. Such a mechanism might account for the differences in frequency of the various tumor types in different strains of animals after administration of acetaminofluorene.

SUMMARY AND CONCLUSIONS

1. Oral administration to rats of small quantities of acetaminofluorene has been followed by the development of a wide variety of tumors in different tissues. Most of the tumors are derived from epithelial cells.

2. Most tissues that give rise to tumors are also the sites of nodular epithelial hyperplasia which is not distinctly neoplastic. No sharp distinction can be made between these hyperplastic nodules and the tumors formed by similar cells.

3. Malignancy can be recognized in some of the tumors by the occurrence of tumor cell infiltration and metastasis.

4. The factors determining the localization of the experimental tumors are not known.

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Distribution and Growth-Potency of Cells in a Transplantable Sarcoma*

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The biological and clinical characteristics of the Crocker mouse sarcoma 180 have in recent years been rather thoroughly studied, especially in relation to experimental procedures. It is common practice among most workers when making new implants to cut the "viable" tissue (usually considered to be the portion having a firm, pearly appearance) of the tumor, freshly removed from the mouse, into fragments about 2 mm. square. With the use of a trochar, one of these cubes is inserted subcutaneously into the axillary region of the host mouse. Within 7 days the implanted fragment grows to a mass of approximately 8 mm. in diameter, at which time it is considered suitable for experimental work or for dissection and reimplantation. If allowed to run its course, at the end of about 2 weeks the tumor attains a diameter of approximately 15 mm., and becomes ulcerous or encrusted. Usually, within 2 to 3 weeks after implantation, the host mouse dies, due presumably to necrosis toxemia; although complete regressions may occur. The weight growth curve of mouse sarcoma 180 so implanted is usually steepest between the fifth and eighth days, and the tumor is non-metastasizing.

In connection with certain experiments requiring a standardization of implanted tumor tissue, it became desirable to make a population and distribution study of the cells comprising this type of tumor at various stages of its growth-cycle, as well as of their growth-potency at such stages. The results of such a study are herein described.

PROCEDURE AND RESULTS

Technic.—When mouse sarcoma 180 is examined in fixed and stained histological preparation, two facts are immediately evident: (a) the cell population of the tumor is heterogeneous as to type, and (b) certain areas of the tumor differ from others in stroma characteristics and in cell distribution. Histological methods do not permit a ready assay of cell-types in experimental tissue at the time of its use, nor do they lend themselves to dependable cell-counting technics. Therefore, a system was sought which would permit sampling of experimental tumor tissue for cell density and cell distribution immediately before implantation. Throughout this study male mice of the inbred Rockland Swiss strain, 18 to 22 gm., were used as hosts.

Tumors were prepared by the trochar method described above. Suspensions were made by removing the whole tumor, of known age, from the mouse, and with the addition of 10 times its volume of Ringer's solution, thoroughly macerating it in a mortar without added abrasive. The resultant suspension was allowed to settle for about five minutes in a 15 ml. tapered test tube, during which time the gross fibrous material was carried down. The supernatant when drawn off in a syringe was found to be a suspension of free cells, which after thorough agitation were in relatively random distribution, and whose population density could therefore be calculated readily from cell counts of samples. It was found that this method of freeing the cells from the fibrous stroma gave a much larger yield of cells than procedures involving the passage of the macerated tissue through cheesecloth or surgical cotton.

An unstained suspension thus prepared, when examined in a hemocytometer under high-dry objective, was found to consist of two primary cell classes: (A) those which appear viable, having clearly defined cell walls, clearly defined nuclear walls, and a characteristic cytoplasmic translucency; and (B) those which appear necrotic, having ruptured or atypical cell walls, pyknotic or atypical nuclear walls, and a characteristically opaque cytoplasm.

The class of cells (A) which we have chosen to designate as "viable" was found further to consist of 5 types: (a) large cells, irregular in contour, often fibroblastoid and fusiform in appearance, with large spherical nuclei which may contain conspicuous nucleoli; (b) small cells, irregular in contour, with small spherical nuclei; (c) polymorphonuclear leucocytes; (d) non-granular cells resembling monocytes or lymphocytes; and (e) cells of groups (a) or (b) in some phase of mitosis.

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The class of cells (B) which we have chosen to designate as "necrotic" cannot in our experience be subdivided as to type. Detritus which early in the study had tended to be confusing, ceased to be troublesome when the above-mentioned grinding and settling technic was employed. Particles in the suspension smaller in diameter than an erythrocyte were not counted as cells. Clumping or agglutination of cells also ceased to be troublesome when the above technic was used.

Shear (7), writing in 1936, stated that he was unable to distinguish living from dead cells in a tumor suspension. Later Belkin and Shear (1), on the basis of staining reaction to neutral red, distinguished three types of cells: (a) cells with diffusely stained cytoplasm; (b) cells with intensely stained nucleus; and (c) cells which remained unstained. They considered types (a) and (c) as viable, and type (b) as dead. Schrek (6) found that a dilute eosin solution added to the suspension aided in differentiating viable from necrotic cells.

We have found that, after some practice, these two arbitrarily defined classes of cells ("viable" and "necrotic") are distinguishable in the unstained condition clearly enough to permit reasonably accurate differential cell counts, and calculation of cell numbers per unit volume of fluid. Although differential counts in many instances were also made of all the cell subvarieties listed above, the data presented in this paper are based principally on counts of "viable" and "necrotic" cells. Results of the complete differential cell counts have not been included here, mainly because of the arbitrary distinction between "large" and "small" cells of the viable class. It was felt that until some functional significance could be attached to these cells which would establish them as distinct types, emphasis on the differential composition of the "viable" cells would be premature. It is of interest that the percentage of mitoses among the viable cells rarely exceeded 1 or 2 per cent during the entire life of the tumor. Since the observations were made on unstained preparations, it is possible that the earliest and latest stages of mitosis were not discernible.

With these methods for assaying the cell population in a given suspension, inocula may be set up whose absolute cell number is controllable and whose differential cell distribution is known approximately. Although a subjective factor enters into the making of count determinations, it has been found during the taking of hundreds of sample counts that estimates by two different observers on identical samples are the same to within plus-or-minus five per cent.

Graphs (A), (B), (C) and (D) in Fig. 1 give the data on the proportion of viable and necrotic cells in suspensions made from whole tumors of various ages, as well as from tumor tissue components of various ages. No attempt has been made to establish the quantitative statistical significance of the data.

Population of Cell Types at Various Stages of Tumor Growth

Suspensions were made of individual whole tumors at intervals in the age range of 2 to 14 days, and counts were made of viable and necrotic cells in samplings of such suspensions. The data so derived are plotted in Fig. 1 (A). It is seen that the young tumor is high in necrotic cells and low in viable cells. Between 6 and 8 days the proportion of viable cells has increased to a maximum, with a corresponding drop in necrotic cells. By 12 days this situation has again reversed itself, and the tumor at this time has the necrotic cell as its most populous type. Macroscopically the 12 or 14 day tumor gives signs of necrosis and ulceration.

The rather peculiar fact that the young tumor is high in necrotic cells warrants some discussion. The classical picture of the body's initial reaction to an implanted tumor fragment is essentially an inflammatory one. Within a few hours after implantation there is a marked aggregation of polymorphonuclear leucocytes in the connective tissue surrounding the fragment. This is succeeded by an infiltration of monocytes and lymphocytes, and then within 24 hours by a multitudinous influx of fibroblasts. The connective tissue surrounding the new implant immediately after inoculation becomes hyaline and subject to infiltration by the above-mentioned succession of wandering white cells, blood cells, fibroblasts, as well as of capillaries, and within a few days appears to become organized into a firm and expanding tissue which comes to constitute the main mass of the tumor. The original fragment does not change appreciably in absolute size or shape (although its cells start undergoing degenerative changes almost immediately after implantation) until about the ninth day when a fusion with surrounding necrotic tissues renders its outlines and structure somewhat amorphous.

The chemical and physiological relationship of the implant to the surrounding connective tissue is not known. Whether the cancer process consists of a multiplication of the cells of the introduced fragment, or whether the surrounding inflammatory connective tissue cells become cancerogenic, or whether two such processes act in combination, remains one of the prime enigmas of cancer biology.

It is quite evident, however, that for mouse sarcoma 180 there is no increase in mass of the originally implanted fragment, and that this fragment, indeed almost immediately after implantation, becomes cytologically degenerative. It is for the latter reason probably that the curve for necrotic cells in Fig. 1 (A) is very high for young whole tumors. At least two-thirds of the total mass of a 2-to-3-day-old tumor consists of the fragment originally implanted.

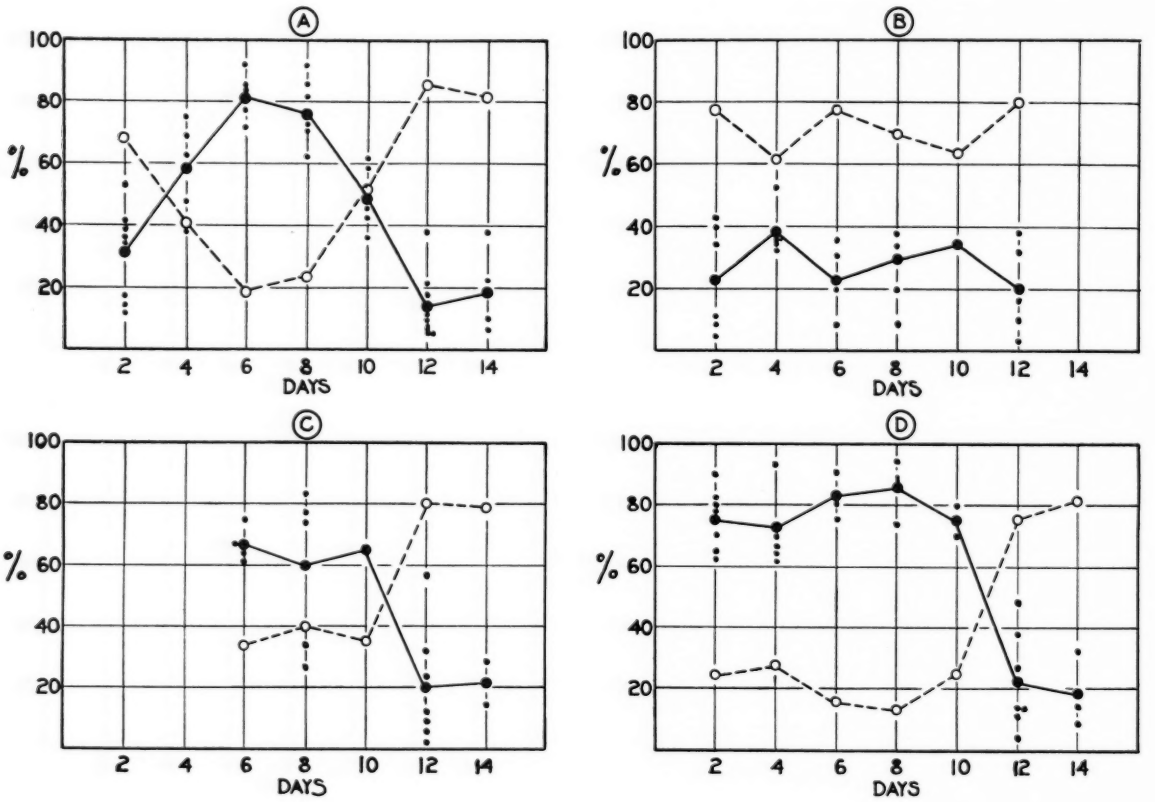

FIG. 1.—Proportion of viable and necrotic cells in suspensions made from tumors of various ages after implantation. Black points represent percentage of viable cells. White points represent percentage of necrotic cells. Each small point refers to a determination made on an individual tumor, and each was derived by counting 50 to 100 cells. The corresponding individual determinations for necrotic cells may be derived as the difference between the viable cell level and 100 per cent. Large points represent the means.

(A)—describes cell proportions in suspensions made from whole tumors; (B)—from central core tissue; (C)—from intermediate zone tissue; (D)—from peripheral zone tissue.

ripheral zone, usually considered to be the tumor's most viable tissue from the standpoint of transplantability, is pearly in appearance and of firm consistency (Fig. 5).

The relative proportion of each of these parts changes markedly throughout the growth history of this transplantable tumor. At 48 hours after implantation about two-thirds of the total tumor mass consists of white caseous core, with the intermediate zone absent or indistinguishable from the

Distribution of Cells within the Tumor at Various Ages

A 7-day-old mouse sarcoma, when bisected equatorially and examined macroscopically, is seen to consist of 3 distinguishable areas. The central core is white and caseous and lacking in vascularity, and constitutes, as we have said, the degenerative remains of the originally implanted fragment. An intermediate zone around the core is somewhat more granular, is vascularized, and its outer boundaries are not well defined. A peripheral zone, which at this early stage is quite hyaline. At 7 or 8 days the central core has not changed appreciably in appearance or in volume, but massive intermediate and peripheral zones have developed. By 12 or 14 days the central core has lost its crisp delineation, as has also the inner boundary of the peripheral zone. An irregular granulation at this time characterizes both the intermediate and peripheral zones, and the tissue mass now appears to resemble that of the earlier intermediate zone, and has become

amorphous, with signs of necrosis and local hemorrhage becoming very evident.

An effort was made to tease out and separate tissues of these 3 zones from tumors of various ages; suspensions were immediately made, and differential cell counts taken. Fig. 1 (B), (C) and (D) present the data so obtained.

It is seen in Fig. 1 (B) that for the central core, the necrotic cell type is most numerous, and that its high level is maintained throughout the period from 2 to 12 days. Viable cells are proportionately low. Determinations at the 14-day level were not made because of the difficulty of dissecting out clearly defined core tissue at this stage.

Fig. 1 (C) shows data on suspensions from the intermediate zone. The intermediate zone does not become well enough defined for successful separation from the other zones until about the sixth day. Hence, data on this zone could be taken only from tumors 6-to-14 days of age. A general similarity between the cell population curves for the intermediate zone and those for the whole tumor (Fig. 1, A) is noted, both types of tissue being highest in viable cells at the 6-8 day stage, and highest in necrotic cells at the 12-14 day stage.

The curves in Fig. 1 (D), derived from peripheral zone suspensions, are also somewhat similar to those shown for the whole tumor (Fig. 1, A) and for the intermediate zone (Fig. 1, C), although there is at least one difference in the early portions of the curve. At 2 and 4 days the number of necrotic cells is significantly lower in the peripheral zone suspension than in the whole tumor suspension. This, on the other hand, is not unexpected in view of the above described central core involvement in the whole tumor data at these early stages. The peripheral tissue has no marked necrotic elements until after about the tenth day, when a general necrosis begins occurring throughout the whole tumor.

Tumor Induction Capacities of Inocula of Known Cell Number

It has been known since the advent of experimental tumor work that tumor cell suspensions may be employed for transplantation purposes. Earliest workers (8) knew that "small" and "large" inocula of tumor suspensions produced proportionately small and large tumors, after a specified interval of time. De Gaëtani and Blothner (2) found that the minimal inoculum necessary to induce grafts of the Ehrlich mouse carcinoma was in the order of 100,000 cells. Kahn and Furth (4) were able to transplant a 1,2-benzpyrene mouse tumor with as few as 50 cells. MacDowell (5) found that inocula of 300 leukemia cells would produce takes in 10 per cent of the mice, and that inocula of 312,000 cells would produce takes in 100 per cent of the mice, while Furth and Kahn (3) have been able to transmit leukemia with a single cell.

The present experiment represents an effort to establish a standardizable relationship between the number of cells in the inoculum and the growth rate of the subsequent tumor mass. Early in the study it became evident that a number of procedural variables had to be tested for possible interference factors. For example, it was found advantageous to standardize the volume of each inoculum (with the exception of certain indicated instances) to 0.1 ml. This was necessary because it was observed that large variations in the absolute volumes of inocula would produce varying local concentrations of cells in the subcutaneous tissues. Thus, a large subcutaneous bleb (0.5 ml.) causes a greater cleavage in the connective tissues than does a small one (0.05 ml.); and, hence, as the liquid of the inoculation vesicle is resorbed, the plaque of cells so deposited in the connective tissue may vary in area and density. A million cells inoculated in a 0.5 ml. volume shows experimentally somewhat less growth than does an equal number of like cells suspended in a 0.05 ml. volume.

A standardization of the site of implantation was also necessary, presumably because of variations in the subcutaneous vascular anatomy or regional resistance factors in the body of the mouse. The procedure finally adopted was to inject the suspension through a No. 23 needle inserted under the epilated skin about ¼ of an inch below the posterior angle of the thoracic basket and about ¼ of an inch off the mid-line. The needle was projected thus subcutaneously to a point in the mid-rib region just under the conspicuous dermal vein in this area. That the inoculation was properly made in each case was easily confirmed by the visibility of this vein riding up over the surface of the resulting bleb.

In preparing suspensions of cells to the required concentration, a series of diluting media was tested. Among these were Tyrode's solution, blood plasma from the tumor donor mouse, supernatant fluid of a centrifuged tumor cell suspension, adult mouse heart juice, Ringer's solution, vitamin-fortified Ringer's solution, a 10 per cent solution of gelatin (7), and glycerin.

Standard Ringer's solution was finally chosen as the simplest diluting medium having no observable adverse or erratic effects on cells in suspension. The acceptability of Ringer's solution was further confirmed by allowing tumor cells so suspended to remain at room temperature for several

hours. Equivalent inocula (of equal volume as well as equal cell number) were removed from such suspensions at hourly intervals. It was found that such inocula did not decrease in potency for tumor induction up to about the third hour. Thereafter, the potency of the inocula began to diminish, due possibly to bacterial contamination or autolysis.

In order to test whether centrifugation affects the potency of the suspended cells, experiments were performed in which some inocula had been subjected to centrifugation and re-suspension, and others not. No growth interference was observed unless centrifugation was repeated 2 or 3 times, and unless the supernatant was wholly replaced by Ringer's solution. Excessive and repeated

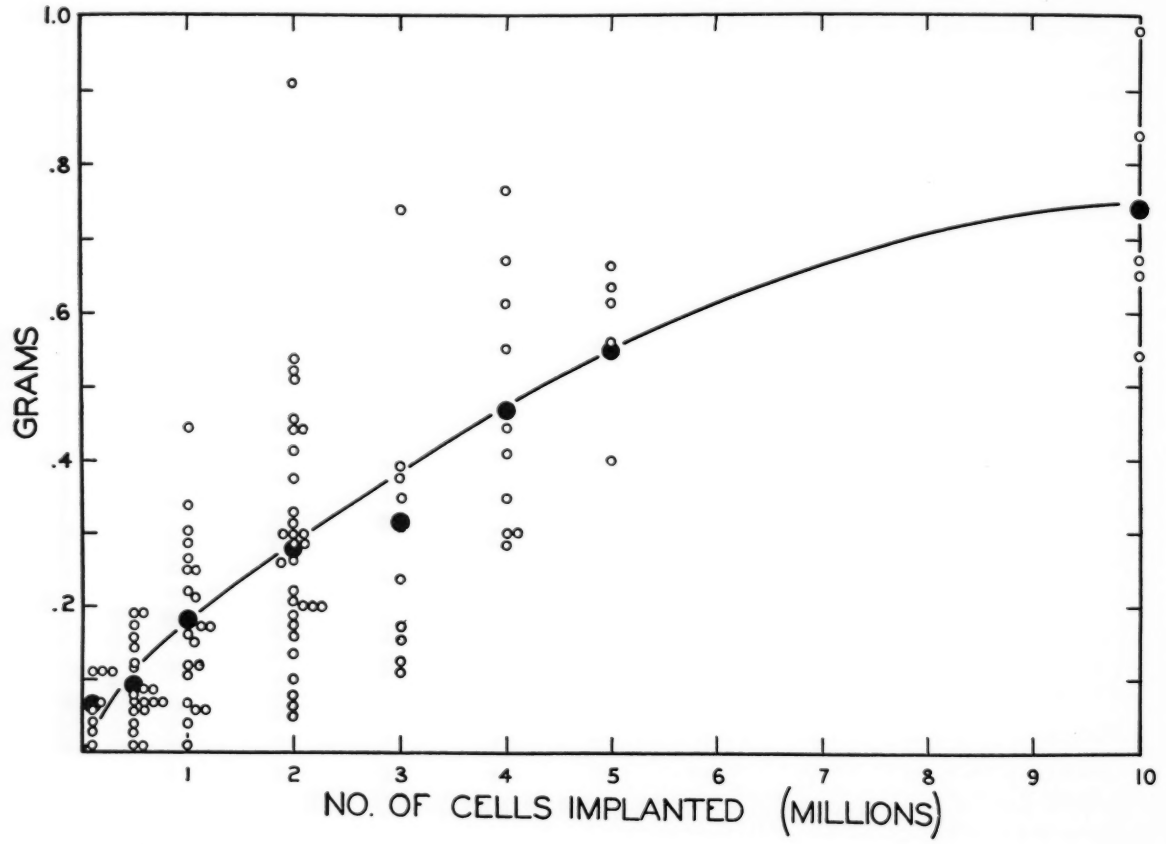

FIG. 2—Weight of tumor at the end of 7 days, plotted against the total number of cells in the inoculum. All inocula were taken from suspensions prepared from 7-day old tumors. Each white point represents one tumor. The black points represent the means.

Insofar as possible, asepsis was observed in dissecting out the tumors and in preparing the suspensions and dilutions; although asepsis in surgical procedures with mice is probably not as critical a desideratum as many workers have supposed. Suspensions known to have been accidentally contaminated during manipulation showed very little difference in resulting tumor growth from those known to be wholly sterile, so long as the time interval between contamination and inoculation was not excessive.

In preparing some of the very dense suspensions required for large-dose inoculations, centrifugation of the primary suspensions, followed by re-suspension in a fraction of the supernatant, was necessary. packing does seem to reduce growth potency of tumor cells to some extent. Whether this is due to mechanical damage to cells, or to other factors involved in centrifugation, is not known. In any case, it was concluded that mild centrifugation and partial decantation of the supernatant is not harmful to the tumor cells and would not increase this general experimental error of the procedure.

The effect of grinding was also studied. Suspensions were made by macerating the tumor in a mortar in the usual way. Portions of such suspensions were subjected to continuous grinding for ten minutes. The growth potency of inocula of equal volume from the lightly and heavily ground preparations did not differ significantly, indicating

that the degree of cell damage, if such occurs in grinding, does not constitute an appreciable experimental hazard.

Employing the procedures suggested by the above discussion, repeated experiments were performed with inocula ranging in cell content from 1000 to 10,000,000. The suspensions were prepared solely from 7-day-old tumors of fragment origin.

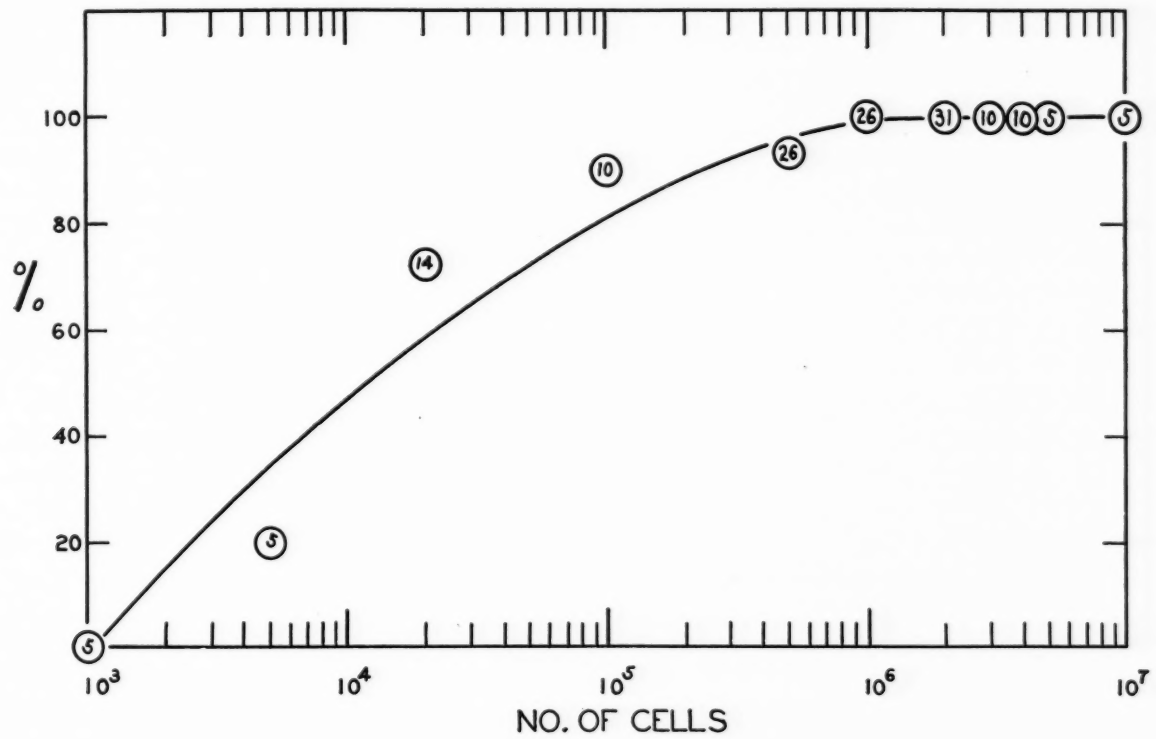

FIG. 3.—Percent of animals showing palpable tumor growths ("takes") at the end of 7 days, plotted against the total number of cells (from 7-day old tumors) in the inoculum. The abscissa range is logarithmic. The number of mice observed at each level is indicated within the circles.

The necessity for choosing donor tumors of standard age is evident from the cell distribution data presented in Fig. 1 (A). The volume of all inocula was 0.1 ml., with the exception of the experiments with inocula containing 10,000,000 cells. In this case, it was necessary to increase the volume of the inoculum to 0.2 ml. At the end of an arbitrarily chosen period of growth after inoculation (168 hours), the mice were killed, and the tumors were wet weighed. Tumor weight at the end of this growth period was taken as a measure of the growth potency of the inoculum. The number of non-takes was recorded.

The data so derived from the range of 100,000 to 10,000,000 cells are plotted in Fig. 2. Although the scatter of individual determinations is considerable at some levels, the trend of the mean curve is clearly evident. In the region of 500,000 to 5,000,000 cells the relationship between number of inoculated cells and resultant tumor growth is essentially rectilinear. The portion of the curve at 10,000,000 was not investigated in sufficient detail to warrant placing reliability on the exact form of the curve in this region.

Data for below 100,000 cells are not plotted in Fig. 2, due to the very low growth at the experimental end-point of 7 days, as well as to the increasing number of non-takes. Mice receiving small inocula (1,000, 5,000 and 20,000 cells) and not showing palpable tumors at the end of 7 days were not killed. A number of these developed tumors after several weeks, thus supporting the contention of Kahn and Furth (4) that some mouse tumors may be transmitted by smaller numbers of cells than was previously supposed. The number of takes above 500,000 was essentially 100 per cent. Below 500,000 the frequency of the takes begins to fall off, reaching zero between 5,000 and 1,000 cells. The percentage of takes as a function of the number of cells in the inoculum is plotted in Fig. 3.

Tumor Induction Potency of Cell Suspensions Made From Tumors of Various Ages

The data plotted in Fig. 4 are derived from a series of 4 experiments in which cell suspensions were made from whole tumors removed 2, 7 and 12 days after implantation of the fragment. Such suspensions were injected at a constant dose of 2,000,000 cells and at a constant volume of 0.1 ml.

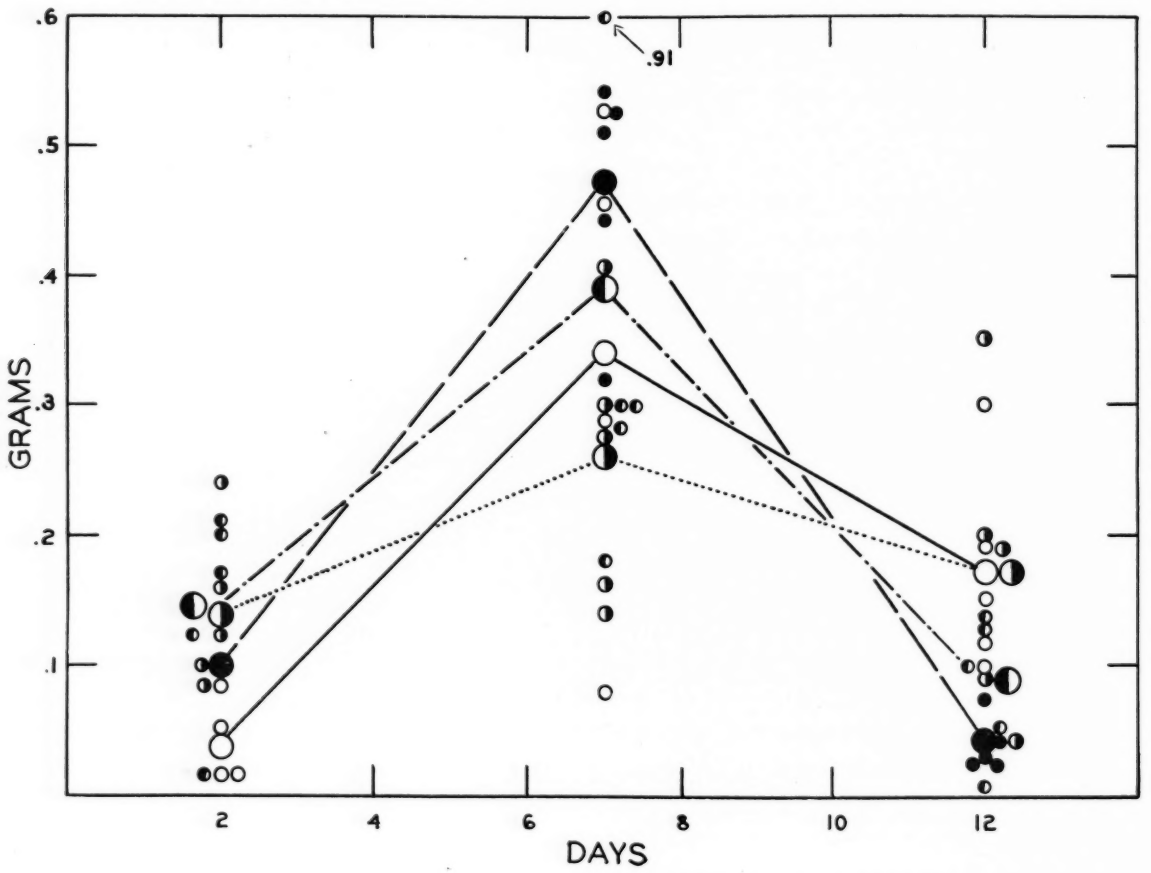

Fig. 4.—Weight of tumor at the end of 7 days after implantation, plotted against the age of the donor tumor from which the inoculation suspension was made. Small points represent individual tumor weights, large points represent the means. Four identically performed experiments are indicated with straight lines connecting the means. All inocula contained a fixed total number of cells (2,000,000).

In preparing these suspensions, a number of tumors of the appropriate age group were often macerated together in order to obtain the required number of cells. Because of this, only total cell counts were made on these suspensions. The differential cell data from Fig. 1 (A) were assumed to be applicable to such pooled-tumor suspensions.

A study of the data presented in Fig. 4 and in Fig. 1 (A) leads to the conclusion that equivalent doses of viable cells (that is, equal absolute numbers of viable cells), regardless of the percentage in the total suspension which they represent, result in approximately equal amounts of tumor growth. For example, it is seen in Fig. 4 that suspensions made from tumors 7 days old were considerably more potent than those from the older or younger tumors. This is not surprising, in view of the data presented in Fig. 1 (A), since 2-day and 12-day tumors have the necrotic cell as their most populous type. It may be calculated from the data in Fig. 1 (A) that a 2,000,000 cell inoculum from a 2-day tumor would contain only about 600,000 viable cells, and one from a 12-day tumor would contain only about 300,000 viable cells. The growth from inocula of such numbers of viable cells corresponds approximately to the growth predictable from Fig. 2 for similarly sized doses of cells taken from 7-day tumor suspensions, known to consist preponderately of viable cells. At the 7-day level of tumor age, an inoculum of 2,000,000 cells would contain about 1,600,000 viable cells. Although the correspondence is not

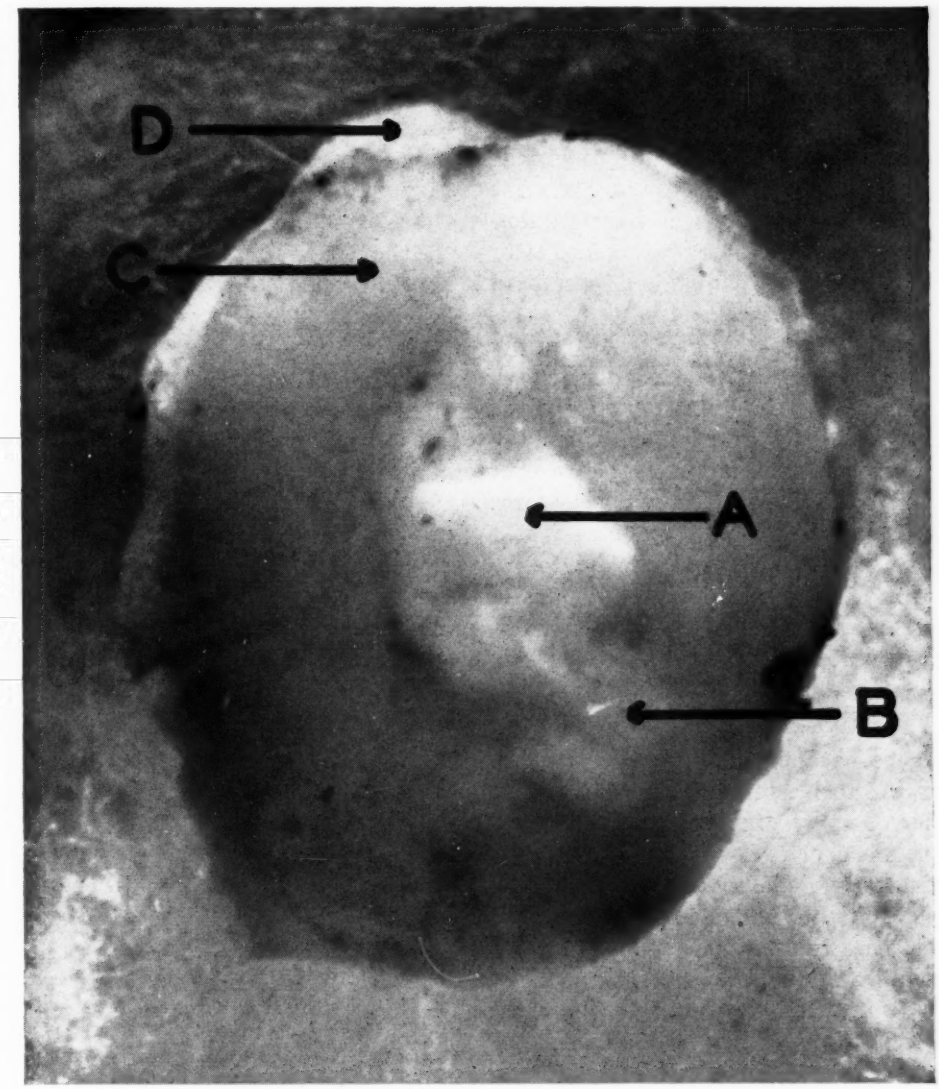

FIG. 5—Photograph of median section through 5-day old mouse sarcoma 180. (A)—central core; (B)—intermediate zone, just becoming evident at 5 days; (C)—peripheral zone; (D)—connective tissue. Mag. × 20.

perfect, it is seen in Fig. 2 that the weight data from such inocula are in the order of magnitude expected from 1,600,000 viable cells.

The correlation between growth potency and the number of what we have chosen to designate as viable cells, thus seems to support the validity of our distinction between "viable" and "necrotic" cells.

Growth potency of suspensions made from isolated tissue areas of the tumor is still under study.

DISCUSSION

It is evident that, with the use of inbred genetically pure mouse strains, the transplantation and growth of experimental tumors has become far more uniform than previously, but that even with such pure strains there is yet a high individual variation in response to tumor implantation.

A study of the distribution of the data in Fig. 2 shows that animals of the same strain, sex, weight, and age, fed on standardized diet, when inoculated with equal numbers of tumor cells of identical origin, and with the use of standardized injection technics, still show a relatively high variation in tumor growth response. This is also evident from the existence of a broad threshold in the region between 100 per cent takes and the beginning of the failure of all the mice to produce tumors. In the failure to observe greater uniformity of tumor

growth response, it seems necessary to assume that a considerable variation in tumor susceptibility exists even in homozygous animals.

The work described in this paper was undertaken as a prelude to a study of adjuvants and repressors of tumor growth. It appeared to us that if one could predict the normal growth behavior of a suspension of type-specified cells, experimentation both on the cells before inoculation and after inoculation could be better controlled than with the usual technic. The data presented in this paper reveal some variables which investigators have been prone to neglect. One variable, for example, which the experimenter must watch especially involves the distribution of cell types within the cancer tissue (either as suspension or fragment) which he implants for further work. On the other hand, even if this important factor is controlled, it is still evident that, due to individual variation among mice, large numbers of animals must be employed to establish the significance of any experimental procedure.

SUMMARY

Mouse tumor cell suspensions prepared by the use of a maceration technic have been studied. It is found that such freshly prepared and unstained suspensions contain a cell population of two distinguishable classes, which have been designated as *viable* and *necrotic*. The viable cells may be further subdivided as to type.

The proportion of viable and necrotic cells in suspensions made from whole tumors is found to vary regularly and significantly as a function of the age of the tumor, in the range of 2 to 14 days. Suspensions from very young tumors (removed 2 to 3 days after implantation) are high in necrotic cells and low in viable cells. The same condition applies to suspensions from old tumors (removed 10 to 14 days after implantation). In suspensions from tumors removed 5 to 8 days after implantation, the viable cells are by far the most numerous.

The central core of the tumor (*i.e.*, the implanted fragment) does not appear to change appreciably in size or shape from the time of implantation to about the tenth day. During the period between implantation and 10 to 12 days the cell distribution in the core tissue does not change significantly, a high and constant level of necrotic cells being found. The intermediate tissue zone is relatively high in viable cells during the 6-to-10 day period, high in necrotic cells thereafter. The peripheral tissue zone is high in viable cells from the second to the tenth day, thereafter becoming high in necrotic cells.

A technic is described for preparing cell suspensions of known cell number and cell type, which when implanted will give fairly predictable tumor growth rates.

The weight of the inoculated tumor after 7 days of growth varies as a function of the number of viable cells introduced. The smallest number of mouse sarcoma 180 cells producing a weighable tumor after 7 days of growth is of the order of 20,000, although inocula containing fewer cells may produce tumors if allowed to remain in the mouse for a longer period of time. The proportion of takes increases as a function of the number of cells introduced, within a specified range.

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Influence of 2,3-Dimercapto Propanol (BAL) on the Induction of Skin Tumors in Mice by 3,4-Benzpyrene

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Among the many agents influencing the rate of induction of skin tumors in mice with a standard carcinogen, hydrolyzing chlor-compounds (1), bromobenzene (2) and unsaturated dibasic acids (3) have been shown to either delay or prevent tumor formation. In attempting to explain why such diverse compounds have a common anti-carcinogenic effect, Crabtree (3) has suggested that their influence is mediated through a disturbance in sulfur metabolism. More specifically, he suggests that these three types of inhibitors react with sulfhydryl groups of the cell by the chemical process of condensation, oxidative coupling, or addition, thus making the sulfhydryl groups unavailable for normal cellular metabolism. Crabtree further postulates that the initial phase involved in the production of skin tumors with a standard carcinogen is the fixation of the carcinogen to the cell through combination with free sulfhydryl groups. If these free -SH groups have been previously blocked by an anti-carcinogen or if the anti-carcinogen competes with the carcinogen for cellular fixation, the carcinogen will be eliminated without producing its characteristic effects.

It was believed that additional evidence to support this theory could be obtained by another approach, namely, to supply the tissue with additional -SH groups which would compete with cellular -SH groups for reaction with the carcinogen. The compound 2, 3-dimercapto propanol (BAL) is known to possess the property of competing with tissue -SH groups for chemicals forming stable -SH complexes (4). Therefore, the effect of BAL on the rate of induction of skin tumors in mice with 3,4-benzpyrene was studied.

EXPERIMENTAL

Albino mice, predominantly males, (National Institute of Health "Swiss") were used in these experiments. The mice were allowed water and Purina special laboratory chow at all times and were housed in an animal room maintained at a uniform temperature. All mice were treated as follows: Three days prior to the first treatment, the hair was clipped from the scapular region. Twice a week, on Monday and Thursday, a 0.3 per cent solution of 3,4-benzpyrene in ether containing 2 per cent liquid paraffin was applied to the interscapular region with a No. 3 brush. In certain of these mice 24 hours after the 3,4-benzpyrene treatment, that is, Tuesday and Friday of each week, 5 per cent BAL in an ointment base consisting of a mixture of "Carbowaxes" was applied over the scapular region. A quantity of 0.08 cc. of the BAL ointment measured from a 0.25 cc. syringe was used for each application.

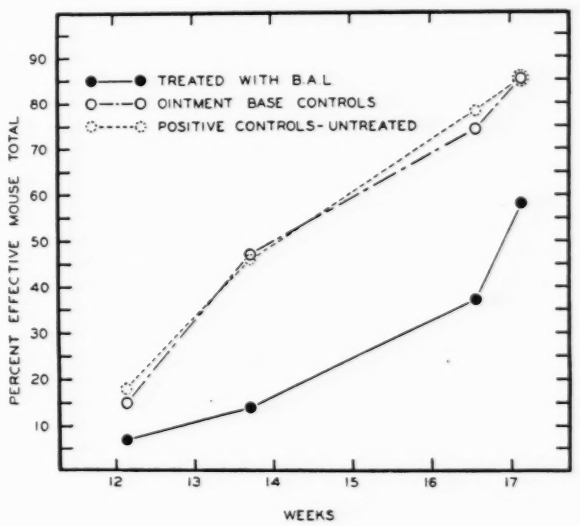

FIG. 1.—Incidence of tumors in mice skin painted with 3,4-benzpyrene.

In a preliminary experiment 70 mice were treated with the 3,4-benzpyrene of which 35 also received applications of 5 per cent BAL ointment. After 14 weeks of treatment, fewer mice receiving the 3,4-benzpyrene plus the BAL showed tumors than did those receiving the carcinogen alone. In order to check the apparently favorable results of BAL treatment obtained in this exploratory experiment and also to rule out any possible physical or chemical effects of the ointment base alone, the following experiment was started: Three com-

parable groups of 55 mice each were selected and were carried on experiment for 17 weeks. In addition to the 3,4-benzpyrene applications which all groups received, one of these groups was treated with BAL ointment as described above. The second group was treated with the ointment base without BAL, while the third group received no further treatment.

RESULTS AND CONCLUSIONS

The final results are shown in Table I. The progression of the experiment can be seen in Fig. 1.

TABLE I: INFLUENCE OF BAL ON INCIDENCE OF SKIN TUMORS AT 17 WEEKS IN MICE TREATED WITH 3,4-BENZPYRENE

Treatment	No. of mice at start	No. survived 17 weeks	No. with tumors	Percentage with tumors
BAL ointment, 5%	55	40	23	57.5*
Control, ointment base	55	34	28	82.4
Control, untreated	55	48	41	85.4

*Reduction in tumor incidence is statistically significant. ($p < 0.01$ by χ^2 test).

The results obtained clearly show the inhibitory influence of BAL on the induction of skin tumors in mouse skin treated with 3,4-benzpyrene. Whether the mechanism of this inhibition is explained by the removal of the carcinogen by -SH groups supplied by the BAL or whether the inhibition is due to a more nonspecific toxic effect of BAL can not be definitely stated. However, the observation of tumor inhibition by this agent deserves mention.

SUMMARY

The incidence of skin tumors in mice painted with 0.3 per cent 3,4-benzpyrene twice weekly for 17 weeks was materially reduced when these mice were treated with 5 per cent BAL ointment 24 hours after each painting with the carcinogen. Only 23 of 40 mice showed tumors after BAL treatment while 41 of 48 mice not treated with BAL showed tumors. Twenty-eight of 34 mice treated with the ointment base developed tumors, thus showing that the physical or physiological effects of constituents of the ointment base were not responsible for the reduced incidence of tumors after BAL.

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